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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 275~276, 1958]

A Synthesis of Pantetheine by the Condensation of Methyl *d*-Pantothenate with 2-Benzylthioethylamine

By Tomiichi SATÔ, Masateru MIYANO and Masanao MATSUI

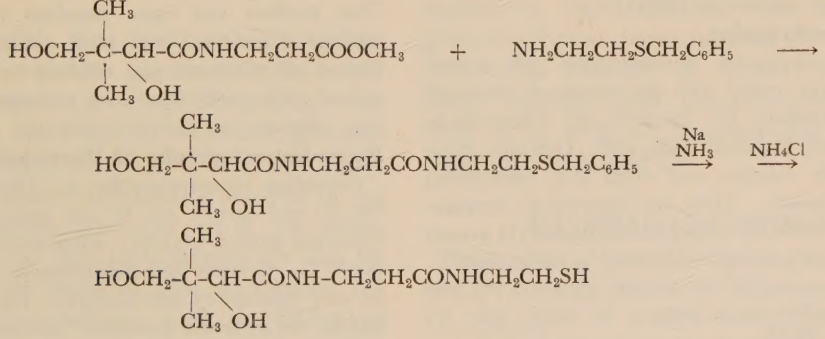
Department of Agricultural Chemistry, University of Tokyo

Received April 15, 1958

Pantetheine (a Lactobacillus Bulgaricus Factor) was obtained by the condensation of pantothenate with benzylthioethylamine following hydrogenolysis of the benzyl group. The method is very convenient and shows a high yield.

Since pantetheine was first recognized as the Lactobacillus Bulgaricus Factor and its structure was synthetically proved to be N-β-pantothenyl-2-mercaptoethylamine by Snell et al. in 1950, several methods for the preparation of pantetheine have been presented.

In 1950, Snell et al.[1] synthesized pantetheine by the condensation of methyl pantothenate with mercaptoethylamine, and the product was found to agree with the natural growth factor in both chromatographic behaviour and biological activity. Thereafter, Wieland et al.[2] synthesized pantetheine by the condensation of ethyl pantothenylcarbonate with cystamine hydrochloride in the presence of 2-N sodium hydroxide, Baddiley et al.,[3,9] King et al.,[4] and Snell et al.[8] by the condensation of pantolactone with N-β-alanyl-β-mercaptoethylamine, Wittle et al.[5] by the condensation of methyl pantothenate (or pantothenoyl azide) with cystamine, or of (−)-pantolactone with N-β-alanyl-β-mercaptoethylamine (or the disulfide), Snell et al.,[7] also, by the condensation of ethyl pantothenate with cystamine (β-mercaptoethylamine), and Funahashi et al.,[10] at first, synthesized pantethine by the condensation of ethyl pantothenate with cystamine, and then obtained pantetheine by reducing it. Walton et al.[6] condensed pantolactone with N-β-alanyl-S-benzyl-2-thiothylamine, and then obtained pantetheine by hydrogenolysis of the benzyl group.



1) E. E. Snell et al., *J. Am. Chem. Soc.*, **72**, 5349 (1950).
2) T. Wieland et al., *Naturwiss.*, **38**, 384 (1951).
3) J. Baddiley et al., *J. Chem. Soc.*, **1952**, 800.
4) T. E. King et al., *J. Am. Chem. Soc.*, **75** (1953).
5) E. L. Wittle et al., *J. Am. Chem. Soc.*, **75** (1953).
6) E. Walton et al., *J. Am. Chem. Soc.*, **76** (1954).
7) E. E. Snell et al., U. S. Patent, 2,680,767 (1954).
8) E. E. Snell et al., U. S. Patent, 2,680,768 (1954).
9) J. Baddiley et al., Brit. Patent, 716,337 (1954).
10) S. Funahashi et al., *J. Agr. Chem. Soc. Japan*, **27**, 772 (1953).

Hereupon, we prepared pantetheine by the route described below. The condensation of the first stage was very smooth and complete, especially under the somewhat excess of methy *d*-pantothenate.

Reducing-splitting of the benzyl group was also very smooth, and the over-all yield was well-nigh quantitative. Furthermore, the product was completely free from any SH-containing impurities or coloured materials without purification. Since methyl *d*-pantothenate can be easily prepared from the commercially available calcium *d*-pantothenate, this method offered another convenience compared with the several methods starting from rather inaccessible pantolactone.

The final product was identified as pantetheine by paperchromatography in a parallel experiment with the authentic specimen.

EXPERIMENTAL

2-Benzylthiothylamine Hydrochloride.[3]

Powdered 2-bromoethylamine Hydrobromide[12] (27.4 grams, 0.134 mole) was added to a solution of benzylmercaptane[11] (18 grams, 0.145 mole) and sodium (6.68 grams, 0.288 mole) in alcohol and the resulting mixture was refluxed in an atmosphere of nitrogen for 1.5 hours. Sodium bromine was filtered off and washed well with alcohol. The filtrate and washing were combined, acidified with concentrated hydrochloric acid, and evaporated to dryness under reduced pressure. 2-Benzylthioethylamine hydrochloride recrystallized from *n*-butanol, melted at 108–111°C.

2-Benzylthioethylamine.

2-Benzylthioethylamine hydrochloride (20 grams, 0.098 mole) was treated with the solution of saturated potassium carbonate, the liberated oily 2-benzylthioethylamine was extracted with ether (100 ml.) three times, and the solution was dried over anyhydrous potassium carbonate. Ether was removed by evaporation and light brown 2-benzylthioethylamine (14 grams) was obtained.

11) Org. Syn., coll. vol. II, (742).
12) *ibid.*, *vol.* 30, 35.
13) G. Toennies et al., *Anal. Chem.*, **23**, 823 (1951).

Methyl pantothenate.

Concentrated sulfuric acid (12.5 grams, 0.127 mole) was dropped into the solution of minimum water containing calcium pantothenate (50 grams, 0.105mole). After the precipitated calcium sulfate was filtered off, the solution was concentrated under reduced pressure, and liberated light brown syrupy pantothenic acid was obtained.

Pantothenic acid was treated with diazomethane prepared by treating nitrosomethylurea with potassium hydroxide in ether, until the solution became slightly yellow. Ether was distilled off, and a light brown syrupy residue was obtained. The yield was 27 grams.

Pantetheine.

Methyl pantothenate (27 grams, 0.116 mole) and benzylthioethylamine (14 grams, 0.084 mole) were dissolved in methanol (80 ml) and the mixture was distilled off, and the mixture was first heated at 100°C for three hours, and then at 150°C for 1 hour on an oil bath without a condenser. Finally, a light-brown syrupy product remained.

Liquid ammonia (about 150 ml.) was added to the product, while the mixture was cooled to about −70°C with dry-ice and acetone, and sodium (11.5 grams, 0.50 mole) in small pieces, was added to the solution with gentle swirling until a deep blue colour persisted in the solution for one hour. Next, ammonium chloride (27 grams, 0.50 mole) was added, and ammonia was removed under room temperature and ordinary pressure. A minimum amount of water was added to the mixture, until the precipitated sodium chloride was perfectly dissolved. The water solution was extracted with *n*-butanol, the solvent distilled off under reduced pressure, and a syrupy product was obtained. This product was again dissolved in a minimum amount of *n*-butanol and after sodium chloride was filtered off, *n*-butanol was distilled by the above procedure. The yield wasal most quantitative. The product obtained was light-brown syrupy state.

Paperchromatography of Pantetheine.

Ascending chromatography on Tôyô Filter Paper No. 50 was carried out by the usual method in the following solvent mixture : *n*-butanol-water-acetic acid (4 : 5 : 1). Cyanide and nitroprusside spray proved to be very satisfactory and sensitive. The above product and/or the authentic specimen[10] showed a R_F value of 0.87.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 277~282, 1958]

Hydrolysis Rate of Insoluble Glucose-Polymer in Dilute Sulfuric Acid

By Tatsuyoshi KOBAYASHI and Yoshio SAKAI

Faculty of Agriculture, Tokyo University of Education

Received April 21, 1958

The purpose of this report is to investigate the hydrolysis rate of insoluble glucose-polymer regenerated when adequate water is added to cellulose dissolved in concentrated sulfuric acid. It has been found that the hydrolysis of this substance is of the first-order in sulfuric acid concentrations ranging from 5 to 40% at the temperature range of 115 to 147°C. The reaction constant is represented as a function of sulfuric acid concentration (C%) and temperature (T°K) by the following empirical equation:

$$k=6.44\times10^{10}\,C^{1.44}\exp\{-25400/(RT)\}.$$

The hydrolysis rate of insoluble glucose-polymer is not so large in comparison of that of cellulose.

INTRODUCTION

In the wood saccharification process with concentrated sulfuric acid, in case of treating cellulosic material with concentrated sulfuric acid (the main-hydrolysis), cellulose converts to glucose-polymers which are both soluble and insoluble in dilute sulfuric acid, although the quantity of insoluble polymer is very small provided the main-hydrolysis proceeds adequately. This polymer is converted into glucose-monomers by the so-called post-hydrolysis process.

Previous work[1] has dealt with the hydrolysis rate of soluble glucose-polymer, but work concening that of insoluble polymer has not yet been clarified. If insoluble glucose-polymer is hydrolyzed at a velosity large enough to complete the post-hydrolysis in the practical retention time and all of the cellulose subjected to the main-hydrolysis can be recovered in the form of glucose-polymer insoluble in dilute sulfuric acid, the following process described here can be established. This process has been suggested by Ichino.[2] After a cellulosic material is mixed with concentrated sulfuric acid, an adequate quantity of water is added to the resulted mixture and the insoluble part that accompanies a small quantity of sulfuric acid is separated from the soluble part; thence, the insoluble part is subjected to the post-hydrolysis with water and the soluble part to the concentration process of sulfuric acid.

This paper which comprises the eighth of a series of articles concerning the wood saccharification process with strong sulfuric acid[1,3] reports on the hydrolysis rates of insoluble glucose-polymer reprecipitated from the sulfuric acid solution of cotton cellulose in dilute sulfuric acid concentrations, ranging from 5 to 40% at temperatures within the range of 115 to 147°C.

MATERIAL AND METHODS

Preparation of Insoluble Glucose-Polymer Sample and Its Average Degree of Polymerization. After 5 g (dry basis) of purified cotton (Pharmacopoeica

1) T. Kobayashi and Y. Sakai, This Bulletin, **22**, 117 (1958).

2) K. Ichino, *Hakkō-kōgaku-zasshi* (*J. Fermentation Technol.*), **30**, 347 (1952).

3) T. Kobayashi, *Mokuzaitōka-shingikai-hōkoku* (*Report of the Wood Saccharification Discussion Committee*), **No. 1**, 27 (1952); **No. 2**, 35 (1953); T. Kobayashi and Y. Sakai, *Ibid.*, **No. 2**, 55 (1953); This Bulletin, **20**, 1 (1956); T. Kobayashi and T. Ito, *Mokuzaitōka-shingikai-hōkoku* (*Report of the Wood Saccharification Committee*), **No. 4**, 5 (1954); T. Kobayashi, K. Mitachi and T. Tsuyuzaki, *ibid.*, **No. 5**, 35 (1956); T. Kobayashi, T. Hirose and K. Mitachi, *ibid.*, 49.

Japonica) was dried in an air-bath, it was treated in a porcelain mortar with 10 g of 72 and 76 % sulfuric acid at room temperature for 30, 40 and 60 min. The temperature of the mass ranged from 28 to 36°C. At the end of this time, water sufficient to result 5 % sulfuric acid in the mixture was added to the mass, and then the sediments were centrifuged, washed in 5, 10, 20 and 40% sulfuric acid and suspended in 100ml. of dilute sulfuric acid of each concentration. The average degree of polymerization (av. D.P.) of insoluble glucose-polymer in the resulted suspension which was subjected to the measurement of the hydrolysis rate ranged from 23 to 125. The conditions for the preparation of the insoluble glucose-polymer samples, the av. D. P. and the yield of the sample are tabulated in Table I.

TABLE I
PREPARATION OF INSOLUBLE GLUCOSE-POLYMER

H_2SO_4 Concn.	Reactn. Time	Reactn. Temp.	Yield	Av. D. P. of Product
%	min.	°C	%	
72	30	28	87	125
		31	82	60
	40	28	80	66
		30	77	64
	50	28	86	50
		28	86	50
	60	30	68	47
		32	56	45
76	30	34	52	41
		36	40	36
	40	33	40	23
		34	52	40

The av. D. P. of the sample was determined by the following procedure. The actual number of end groups of insoluble glucose-polymer in the suspension was determined by the alkaline hypoiodite method of Willstätter and Schudel[4]. The potential number of end groups of the sample was determined by the same method after the insoluble part in the suspension was quantitatively saccharified according to the method described later. The potential number of the end groups was divided by the actual number of end groups and the quotientd was substituted for the av. D.P. of sample.

Measurement of Hydrolysis Rate. The method by which the experiment was carried out was similar to that of Saeman.[5] A sample (2 ml. of the suspension described above) was transferred to a small glass tube which was subsequently sealed in a blow lamp. This glass bomb was heated in a paraffin bath with a temperature regulator at 115, 130 and 147°C. After it was heated for a certain period, it was opened and the content was transferred to a centrifuge tube, and then the hydrolysis residue was centrifuged and washed four times in sulfuric acid. The sediment washed was quantitatively saccharified[6] and the potential reducing sugar in the sediment was determined as follows: When the volume of the sediment was less than 1 ml., it was made up to 1 ml. with 5 % sulfuric acid. To the sediment in the centrifuge tube, 4 ml. of 86 % sulfuric acid was added (this acid and water maintained by solid, consitute about 75 % sulfuric acid). Then, it was mixed thoroughly with a stirring rod and placed in a bath at 30°C. This temperature was maintained for 45 min. while the mixture was stirred at an interval of about 10 min. After the total time of 45 min., the mixture to which 67 ml. of water was added was heated at 100°C for 3 hrs., the residue was then filtered off and the reducing sugar in the neutralized filtrate was determined, the new reagent of Somogyi[7] being used.

The extent of hydrolysis was expressed as grams of the residual potential reducing sugar in the hydrolysis residue per gram of potential reducing sugar in the starting material. The rate of hydrolysis was computed from a straight line, logarithm of the extent of hydrolysis *versus* time, in minutes.

RESULTS

The extents of hydrolysis of insoluble glucose-polymer at different times under various conditions are given in Figs. 1 to 4. The straight lines fitted by the method of least squares indicate that the reactions were of the first-order. The slope of the straight line multiplied by −2.303 gave a first-order reaction constant. The first-order reaction constants for hydrolysis of insoluble glucose-polymers which have different chain lengths in 5, 10, 20 and 40% sulfuric acid at 115, 130 and 147°C, respectively, are shown in Table II.

4) R. Willstätter and G. Schudel, *Ber.* **51**, 780 (1918).
5) J. F. Saeman, *Ind. Eng. Chem.*, **37**, 43 (1945).
6) J. F. Saeman, J. L. Bubl and E. E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **17**, 35 (1945).
7) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1954).

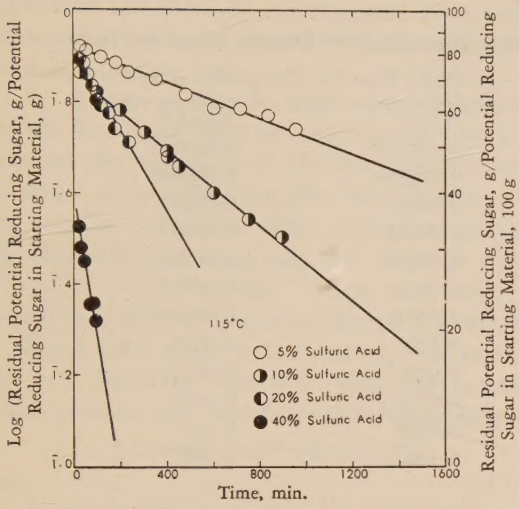


FIG. 1. Hydrolysis of Insoluble Glucose-Polymer in 5, 10, 20 and 40 % Sulfuric Acid at 115°C.

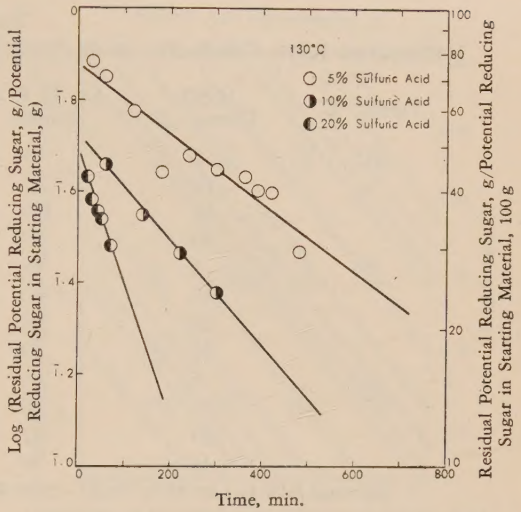


FIG. 2. Hydrolysis of Insoluble Glucose-Polymer in 5, 10 and 20 % Sulfuric Acid at 130°C.

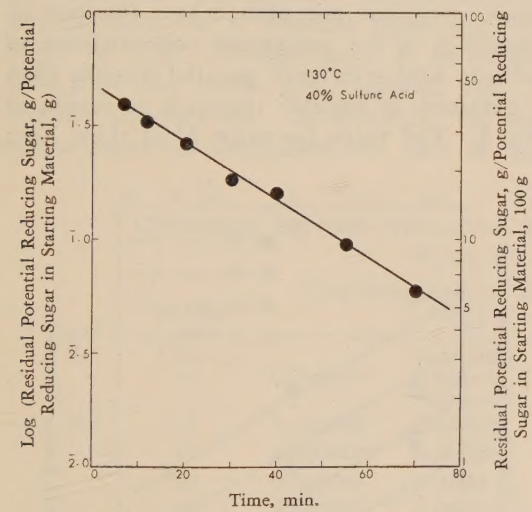


FIG. 3. Hydrolysis of Insoluble Glucose-Polymer in 40 % Sulfuric Acid at 130°C.

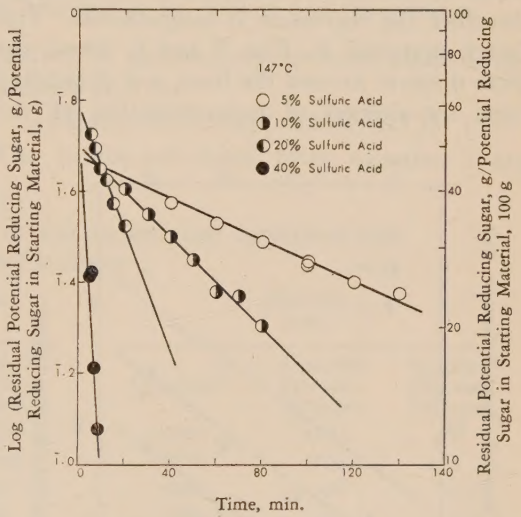


FIG. 4. Hydrolysis of Insoluble Glucose-Polymer in 5, 10, 20 and 40 % Sulfuric Acid at 147°C.

DISCUSSION AND CONCLUSION

The interpretation of data in Table II by analysis of covariance[8] shows that the hydrolysis rates of insoluble glucose-polymers of which the av. D. P. ranges from 23 to 125 are not affected by the initial chain length, since the regression coefficient of the logarithm of hydrolysis rate upon the average chain length is 0.000173, variance due to regression is 0.000172, and the variance about regression is 6.42; when the ratio of variance due to re-

8) G. Taguchi, Zikken-keikaku-hô (1), the Chemical Society of Japan, 1952, pp. 75~91.

TABLE II

HYDROLYSIS RATE CONSTANTS OF GLUCOSE-POLYMER INSOLUBLE IN DILUTE SULFURIC ACID

Temp. °C	H_2SO_4 Concn. %	Av. D. P. of Sample	1st-Order Reaction Constant k (min.$^{-1}$)	
			Obsd.	Calcd.*
115	5	45	0.000441	0.000335
	10	41	0.000950	0.000902
	20	125	0.00204	0.00243
	40	23	0.00652	0.00653
130	5	64	0.00173	0.00131
	10	40	0.00265	0.00352
	20	36	0.00671	0.00948
	40	50	0.0295	0.0255
147	5	66	0.00512	0.00513
	10	60	0.0115	0.0139
	20	47	0.0304	0.0373
	40	50	0.168	0.100

* Calculated from $k = 6.44 \times 10^{10} C^{1.44} \exp\{-25400/(RT)\}$

gression to variance about regression is calculated, an extremely small value is obtained; therefore the regression is insignificant. This fact is apparent in Figs. 5 and 6, where the plots disperse around the lines, not depending upon the degrees of polymerization of the starting material.

When the logarithms of the first-order reaction constants are plotted as a function of logarithm of the percentage concentration of sulfuric acid, a series of parallel straight lines is obtained in regular intervals as shown in Fig. 5. The value for slope M of these lines

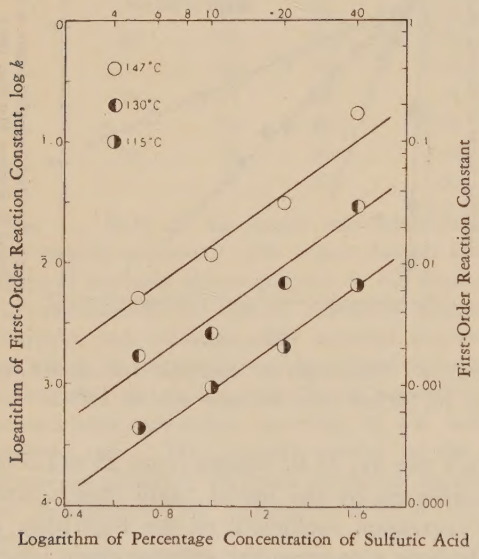


FIG. 5. Relation of First-Order Reaction Constant to Percentage Concentration of Sulfuric Acid in Hydrolysis of Insoluble Glucose-Polymer at Different Temperatures.

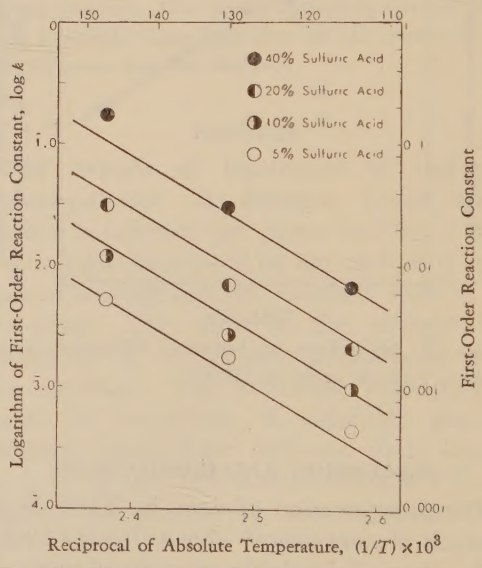


FIG. 6. Relation of First-Order Reaction Constant to Temperature in Hydrolysis of Insoluble Glucose-Polymer with Sulfuric acid of Different Strengths.

describes the effect of sulfuric acid concentration on hydrolysis of insoluble glucose-polymer and is equal to 1.44.

As shown in Fig. 6, when the logarithms of the first-order reaction constants are plotted against the reciprocal of absolute temperature, a series of parallel straight lines is obtained in regular intervals. The activation energy is obtained by multiplying the slope of these lines by -2.303×1.986. When this slope is equal to -5925, activivation energy is 25400cal.

These facts show that equation 1 described by J. F. Saeman[5] fits well with the present data without any modification being necessary.

$$k=H\cdot C^{M}\cdot\exp\{-E/(RT)\}$$

or,

$$\log k=\log H+M\cdot\log C-\{E/(2.303\,RT)\} \quad (1)$$

where k is the first-order reaction constant, H a constant, C the percentage concentration of sulfuric acid, M a constant describing the effect of acid concentration, E activation energy, R the molar gas constant, and T absolute temperature. Accordingly, the empirical equation describing the rate of hydrolysis of glucose-polymer insoluble in dilute sulfuric acid is:

$$k=6.44\times10^{10}C^{1.44}\exp\{-25400/(RT)\} \quad (2)$$

The values calculated from equation 2 are

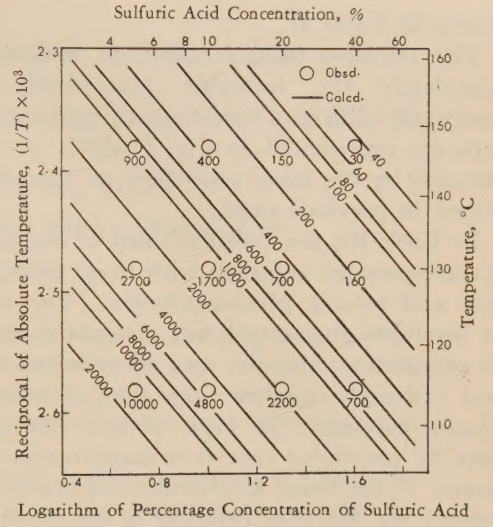


FIG. 7. Retention Time Completing the Hydrolysis of Insoluble Glucose-Polymers Expressed as Function of Temperature and Sulfuric Acid Concentration.

TABLE III
COMPARISON BETWEEN HYDROLYSIS RATES OF CELLULOSE, AND INSOLUBLE AND SOLUBLE GLUCOSE-POLYMERS

Temp. °C	Sulfuric Acid Concu. %	First-Order Reaction Constant min^{-1}			Half-life min.		
		Cellulose*	Insoluble Glucose-Polymer	Soluble** Glucose-Polymer	Cellulose*	Insoluble Glucose-Polymer	Soluble** Glucose-Polymer
100	5	0.0000199	0.000169	0.0260	35000	4100	27
	10	0.0000532	0.000458	0.0466	13000	1500	15
	20	0.000143	0.00124	0.135	4800	560	5.1
	40	0.000381	0.00335	0.981	1800	200	0.7
115	5	0.000110	0.000335	0.109	6300	2000	6.4
	10	0.000294	0.000902	0.195	2400	770	3.6
	20	0.000785	0.00243	0.563	880	290	1.2
	40	0.00210	0.00653	4.26	330	110	0.2
130	5	0.000607	0.00131	0.454	1100	530	1.5
	10	0.00163	0.00352	0.813	430	200	0.9
	20	0.00387	0.00948	2.35	180	73	0.3
	40	0.0116	0.0255	13.3	60	27	0.05

* Calculated from $k=1.57\times10^{14}C^{1.42}\exp\{-34000/(RT)\}$[9]

** Calculated from $k=1.61\times10^{15}\exp[-1.46H_0-\{28400/(RT)\}]$[1]

9) T. Kobayashi and Y Sakai, in T. Asai (Editor), Kôboriyôkogyô, Kyôritsu-shuppansha, Tôkyô, 1957, p. 188

shown in Table II.

The retention times required to hydrolyze completely the insoluble glucose-polymer under the different conditions of post-hydrolysis are represented in Fig. 7 which is constructed by the same procedure as that described in previous work.[1)]

In Table III, the hydrolysis rate of insoluble glucose-polymer contrasts with those of cellulose and soluble glucose-polymer. The rate of insoluble glucose-polymer was not so large in comparison with the rate of cellulose and was extremely smaller than that of soluble glucose-polymer. In view of this result, it may be concluded that it is inappropriate in course of the wood saccharification process to subject the degraded cellulose in the insoluble form to post-hydrolysis.

In Figs. 1 to 4 none of the straight lines reach the initial concentration when they are extrapolated to zero time. From this fact, it may be assumed that the readily hydrolyzed fraction exists in the insoluble glucose-polymers prepared by the degradation of cellulose. It is desirable that further studies concerning the problem of such a fraction should be carried out in connection with the chain length distribution and hydrolysis rate of glucose-polymer. Provided the insoluble fraction easy to hydrolyze is collected in high yield, the process involving the dilute acid-hydrolysis of insoluble glucose-polymer followed by the separation of precipitated fraction from sulfuric acid will be able to be established.

Acknowledgment We wish to express our appreciation to members of the Wood Saccharification Committee for their helpful suggestions during the course of this work. Also, we wish to thank Mr. K. Iizuka for his helpful assistance during this experiment.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 283~286, 1958]

Enzymatic Resolution of Racemic Amino Acids

Part VII. Availability of the Acyl Derivatives of Lysine for the Growth of Rats

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Received April 26, 1958

The present paper is concerned with the availability of the acyl derivatives of lysine for the growth of young rats in the course of studying the enzymatic resolution of DL-lysine with mold acylase. The enzymatic resolution of DL-lysine to optically-active L and D-isomers was carried out in either of the following two ways, namely, the asymmetric hydrolysis of diacetyl-DL-lysine or that of ε-benzoyl-α-acetyl-DL-lysine.

The oral administration of ε-acetyl-L-lysine to rats fed on the lysine-deficient diet supported the growth of young rats at a rate approximately two-thirds of that observed when L-lysine was supplied. ε-Benzoyl-L-lysine proved to be quite ineffective while diacetyl lysine showed a slight but insignificant increase in body weight.

Neuberger and Sanger[1] showed that ε-acetyl-L-lysine can replace the free L-lysine when fed to growing rats resulting in good growth as those subjected to the L-lysine diet by oral administration or by intraperitoneal injection. Paik and Greenstein[2] et al., have recently reported that supplementation of the lysine-deficient diet with ε-acetyl-L-lysine leads to the growth of rats which is in accord with the earlier observations of former investigators and have proved the presence of an ε-acylase in the rat tissues, especially in the kidney, capable of hydrolytic deacetylation of ε-acetyl-L-lysine which is distinct from the renal α-acylase.

The present authors[3,4,5] have been engaged in a series of studies on enzymatic resolution of racemic amino acids by using mold amino acid acylase. The present paper concerns the enzymatic resolution of DL-lysine and the availability of ε-acyl derivatives of L-lysine for the growth response in rats. Acyl derivatives of L-lysine and L and D-isomers of lysine were obtained by enzymatic resolution procedure with the mold acylase of *Penicillium* as described previously.[4,5] The resolution of DL-lysine was undertaken in either of the following two ways, namely, by asymmetric hydrolysis of diacetyl-DL-lysine or that of ε-benzoyl-α-acetyl-DL-lysine. In these procedures ε-acetyl-L-lysine and ε-benzoyl-L-lysine were obtained as intermediate products.

1) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).
2) W. K. Paik, L. Bloch-Frankenthal, S.M. Birnbaum, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **69**, 56 (1957).
3) K. Michi and H. Nonaka, This Bulletin, **19**, 153 (1955).
4) K. Michi and H. Tsuda, This Bulletin, **21**, 18 (1957).
5) K. Michi and H. Tsuda, This Bulletin, **21**, 235 (1957).

EXPERIMENTAL

I. Resolution of Diacetyl-DL-lysine

Diacetyl-DL-lysine. Diacety-DL-lysine was prepared as usual, namely, by acetylation of DL-lysine·2HCl (0.1 Mol) in 2N NaOH (0.3 Mol) with acetic anhydride (0.4 Mol) and the simultaneous addition of 2N NaOH (0.4 Mol) in an ice-bath. Diacetyl-DL-lysine was crystallized from an alcohol-acetone mixture. Yield 80% of theo., m. p. 141°C.

Anal. Found: N, 11.87. Calcd. for $C_{10}H_{18}O_4N_2$: N, 12.10%.

ε-Acetyl-DL-lysine. Fifteen g of diacetyl-DL-lysine was dissolved in 400 ml of water and neutralized by the addition of 3.5 g of calcium carbonate. The solution was incubated with the addition of *Penicillium* acylase which was prepared from 300 g of molded bran as described previously.[4] After two days' incubation,

the reaction mixture was treated with oxalic acid so as to remove calcium. The filtrate was evaporated in vacuo to a small volume and treated with an excess of hot absolute alcohol. ε-Acetyl-L-lysine was crystallized out rapidly from the solution. After being allowed to stand in a refrigerator overnight, the precipitate was filtered and washed with absolute alcohol. Another crop of ε-acetyl-L-lysine was obtained from the filtrate. Yield 5.5 g (90 % of theo.) Recrystallized compound from dilute alcohol gave m. p. 244°C, $[\alpha]_D^{19}+22.0°$. (c 2, 5N HCl).

Anal. Found: N, 14.62. Calcd. for $C_8H_{16}O_3N_2$: N, 14.89 %.

L-Lysine. ε-Acetyl-L-lysine (5 g) added with 2N HCl was refluxed for two hours. L-Lysine-2HCl was obtained from the evaporated residue of the digest. Yield 4.9 g (84.5 % of theo., over-all yield 70% from diacetyl lysine). Recrystallized L-lysine·2HCl gave m.p. 195–198°C, $[\alpha]_D^{19}+17.26°$ (c 4, 5N HCl), $[\alpha]_D^{19}+25.86°$ (for free L-lysine).

Anal. Found: N, 12.52. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77 %.

D-Lysin. The filtrate of ε-acetyl-L-lysine and the washings were evaporated to dryness and dissolved in 50 ml of water. The solution which possesed a ninhydrin reaction was adjusted with 0.5 ml of 6N HCl to pH 1 and passed through a 20 cm column of Dowex 50×4 (Na^+) in order to remove ε-acetyl-L-lysine. The filtrate which gave a negative ninhydrin reaction was acidified with 6N HCl to a 2N HCl content and refluxed for two hours. When the digest was evaporated to dryness in vacuo, D-lysine·2HCl was obtained with the addition of alcohol. Yield 5 g (70 % of theo.) The recrystallized compound gave m. p. 195–198°C and $[\alpha]_D^{18}-17.20°$ (c 4, 6N HCl).

II. Resolution of ε-Benzoyl-α-acetyl-DL-lysine

ε-Benzoyl-α-acetyl-DL-lysine. ε-Benzoyl-α-acetyl-DL-lysine was prepared by acetylation of ε-benzoyl-DL-lisine with acetic anhydride and 2N NaOH as usual. The separated oily ε-benzoyl-α-acetyl-DL-lysine obtained from the reaction mixture by the addition of 6N HCl was brought to crystallization in a refrigerator. The compound which was collected on filter by suction was washed with cold water. Recrystallization was carried out by dissolving the compound in dilute sodium hydroxide in a cold bath and precipitation with the addition of 2N HCl. The purified compound gave m. p. 145°C.

Anal. Found: N, 9.45. Calcd. for $C_{15}H_{20}O_4N_2$: N, 9.58 %.

ε-Benzoyl-L-lysine. ε-Benzoyl-α-acetyl-DL-lysine (60 g) was dissolved in water added with 2N NaOH. The solution which showed pH 7 was incubated with *Penicillium* acylase prepared from 800–1000 g of molded bran at 37°C for two days. ε-Benzoyl-L-lysine precipitated during incubation was filtered and washed with cold water and then with alcohol. Yield 22 g (85 % of theo.) m.p. 266°C, $[\alpha]_D^{18}+19.48°$ (C 2.4, 6N HCl).

The filtrate was separated for the preparation of D-lysine.

L-Lysine. ε-Benzoyl-L-lysine (20 g) was refluxed with 6N HCl (200 ml) for eight hours. Yield 14 g (80 % of theo.) Recrystallized L-lysine·2HCl gave m.p. 198°C and $[\alpha]_D^{18}+17.46°$C (C 4, 6N HCl), $[\alpha]_D^{18}+26.07°$ (for free base).

Anal. Found: N, 12.47; Cl, 32.30 %. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77; Cl, 32.39 %.

D-Lysine. The filtrate separated from ε-benzoyl-L-lysine was evaporated to about 70 ml and the solution acidified with 2N HCl was extracted with ethylacetate as described by Chibata et al.[6] The ethylacete layer was separated from the ninhydrin reaction-positive solution. The combined ethylacetate solution was evaporated and the residue was refluxed with 6N HCl (200 ml) for eight hours. The purified D-lysine·2HCl gave m. p. 198°C and $[\alpha]_D^{18}-17.38°$ (C 4, 6N HCl), $[\alpha]_D^{18}-26.04°$ (for free base). Yield 11g (63% of theo.)

Anal. Found: N, 12.42; Cl, 32.43 %. Calcd. for $C_6H_{16}O_2N_2Cl_2$: N, 12.77; Cl, 32.39.

III. Animal Experiment.

Male weanling rats of the Wistar strain with an average weight of 50 to 55 g were used. The animals were divided into seven groups, each consisting of six to eight rats. The animals were housed in individual cages equipped with raised screen bottoms. They were fed ad libitum and weighed twice a week during the experimental periods of four weeks.

As shown in Table I, the basal lysine deficient diet (Diet I) consisted of wheat flour, Yakifu (dried gluten), soybean oil, salts and known vitamins. The rations were supplemented with 0.6 % of DL-lysine (Diet II), 0.3 % of L-lysine (Diet III), 0.3 % of D-lysine (Diet IV), 0.258% of ε-acetyl-L-lysine (Diet V), 0.342 % of ε-benzoyl-L-lysine (Diet VI) and 0.63 % of diacetyl-DL-lysine (Diet VII) in equivalents to a supplement of L-lysine and DL-lysine. The nitrogen content of the rations was maintained at the same level by appropriate addition of glycine and a small amount of glucose

6) I. Chibata, S. Yamada and S. Yamada, This Bulletin, **20**, 174 (1956).

Table I Composition of Diets

Ingredient	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI	Diet VII
Wheat flour[1]	84 %	84 %	84 %	84 %	84 %	84 %	84 %
Yakifu[2]	6	6	6	6	6	6	6
Salt mixture	4	4	4	4	4	4	4
Soybean oil	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Vitamin A, D, E oil[3]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.15	0.15
DL-Lysine·2HCl		0.6					
L-Lysine·2HCl			0.3				
D-Lysine·2HCl				0.3			
ε-Acetyl-L-lysine					0.258		
ε-Benzoyl-L-lysine						0.342	
Diacetyl-DL-lysine							0.63
Glycine	0.42		0.21	0.21	0.21	0.21	
Glucose	0.43	0.25	0.34	0.34	0.38	0.30	0.22
	[4] 100.00	100.00	100.00	100.00	100.00	100.00	100.00

1) N, 1.48 %.
2) Commercial wheat gluten, N, 5.31 %.
3) 2000 I. U. of vitamin A, 400 I. U. of vitamin D and 10 mg of vitamin E contained in 0.5 g of oil.
4) Calculated total N % of amino acids and protein: N, 1.64 % in 100 g of diet.

Table II Vitamin Supplements

	mg per kg of diet
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine hydrochloride	5
Nicotinic acid	25
Calcium pantothenate	25
p-Aminobenzoic acid	300
2-Methyl-1, 4-naphthoquinone	2
Biotin	0.1
Folic acid	0.2
Vitamin B_{12}	0.02
Inositol	100

was added to make the contents up to 100 %.

RESULTS AND DISCUSSION

Several investigators have shown that the nutritive value of wheat flour protein could be improved by the addition of lysine together with other essential amino acids. Sure[7] has shown that the quality of milled wheat flour (86.4 %) diet is improved by the supplement of 0.4 % of L-lysine, 0.2 % of DL-threonine and 0.7 % of DL-valine. The present authors have employed a wheat flour diet supplemented with dried gluten (Yakifu) as a substitute for supplementation with pure essential amino acids, such as DL-threonine.

As shown in Table III, the growth response in rats supplemented with L-lysine, DL-lysine and ε-acetyl-L-lysine was definitely improved over that of the negative control group (Diet

Table III Growth Responses of Young Rats Fed on Experimental Diets

Diet No.	No. of animals	Mean gain in weight in a 28-day period and probable error (g)	Nature of diet
I	8	10.6±1.37	Negative control
II	7	48.4±3.30	DL-Lysine diet
III	6	45.7±3.54	L-Lysine diet
IV	7	10.9±1.89	D-Lysine diet
V	6	31.7±1.49	ε-Acetyl-L-lysine diet
VI	6	10.8±1.16	ε-Benzoyl-L-lysine diet
VII	8	14.3±1.16	Diacetyl-DL-lysine diet

7) B. Sure, *J. Nut.*, **50**, 235 (1953).

I). On the L-lysine diet the average growth rate was 45.7 g±3.5 for four weeks or a 1.6 g gain per day, on the DL-lysine diet 48.4 g± 3.3 for four weeks or a 1.7 g gain per day and on the ε-acetyl-L-lysine diet 31.7 g±1.5 for four weeks or a 1.1 g gain per day. Paik et al. have recently reported a similar growth response in rats by employing a more complete chemically defined-diet. Animals fed on the D-lysine diet and ε-benzoyl-L-lysine diet showed approximately the same average gains, 10.9 g±1.9 and 10.8 g±1.2, respectively, and actually no improvement in growth was observed when compared with 10.6 g±1.4 of the control group without supplement. Those fed on diacetyl-DL-lysine diet showed a slight but insignificant increase in body weight.

The availability of ε-acetyl-L-lysine for the growth of rats was confirmed to be in accord with the results of American and English investigators. ε-Acetyl-L-lysine is not quite so effective in replacing L-lysine in the diet so well as described by Paik et al. Although ε-lysine acylase and α-amino acid acylase have been found in the tissues of rat by Greenstein et al., diacetyl-DL-lysine almost failed to improve the lysine-deficient diet. However, a slight increase of body weight over that of the lysine-deficient group exhibits a very slow inversion of diacetyl-DL-lysine to L-lysine. ε-Benzoyl-L-lysine was quite ineffective in replacing L-lysine. It is supposed that the derivative is not absorbed by the alimentary tract because of its insolubility in water or digestive juice. Optically-active L-lysine had the best availability and D-lysine was completely ineffective.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 287~293, 1958]

Sulfhydryl Groups in Crystalline Sweet Potato β-Amylase

Part I. Interrelation between Residual Activity and Remaining –SH Groups in Oxidized β-Amylase

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By means of amperometric mercurimetric titration, the –SH groups of native and oxidized sweet potato β-amylase have been determined.

Although eight titratable –SH groups were found in the native enzyme molecule, no distinction between essential and non-essential –SH groups was observed. A partially active enzyme after treatment with *o*-iodosobenzoate was crystallized by salting out with ammonium sulfate, and only an extensively oxidized one from water upon dialysis. The oxidized enzyme did not restore its activity upon treatment with sodium thioglycolate. Oxidation by iodine was also carried out from which it was presumed that the oxidation of the enzyme either by *o*-iodosobenzoate or by iodine does not proceed through the "all or none" mechanism, and also that the essentiality of –SH groups would rather be indirect.

It has been well established by many workers,[1~3] that free –SH groups are responsible for β-amylase action.

Concerning sweet potato β-amylase, Balls et al.[4] concluded that all the S–S and –SH sulfur is masked and that a large proportion of it exists as –SH. They also determined the content of cystine plus cysteine after Sullivan et al.,[5] and it was found to be 0.79 per cent. This corresponds to the value of ten –SH per mole enzyme.

Englard et al.[2] have studied the interaction of sweet potato β-amylase with various sulfhydryl reagents concluding that the oxidation of the essential –SH groups proceeds through an intramolecular reaction. In the same course of study they showed that when activity was 95 per cent inhibited as a result of *o*-iodosobenzoate treatment, 75 per cent of the total –SH content is still titratable after denaturation with Duponol PC. This fact indicates that only a portion of –SH groups is essential for enzyme activity. However, the values obtained by them amounted up to 38 moles and 48 moles –SH per mole for the oxidized and unoxidized enzymes, respectively. These values are considered too high as compared with the data obtained by Balls et al.

French[6] et al. have studied the action mechanism of β-amylase, using a labelled substrate, and concluded that this enzyme has only one active center in spite of the fact that on the average, four maltose molecules are removed each time the enzyme attacks an amylose chain.

This article was undertaken to elucidate the more accurate number of –SH groups contained per mole of sweet potato β-amylase and the number of the essential one among them.

1) C. E. Weill and M. L. Caldwell, *J. Am. Chem. Soc.*, **67**, 212 (1945).

2) S. Englard, S. Sorof and T. P. Singer, *J. Biol. Chem.*, **189**, 217 (1951).

3) M. Ito and M. Abe, *J. Agr. Chem. Soc. Japan*, **27**, 762 (1953).

4) A. K. Balls, R. R. Thomson and M. K. Walden, *J. Biol. Chem.*, **173**, 99 (1948).

5) M. X. Sullivan et al., *ibid.*, **145**, 621 (1942).

6) J. M. Bailey and D. French, *J. Biol. Chem.*, **226**, 1 (1957).

MATERIALS AND METHODS

β-Amylase was prepared by the authors' modification of the method of Balls et al. The thrice recrystallized sample, which had the specific activity of 1150 units,[7] was dialysed against distilled water and a stock solution of 8640 units per ml was used throughout.

Ovalbumin was prepared by the method of Kekwick and Cannan[8] and was twice recrystallized.

o-Iodosobenzoic acid was prepared according to Hellerman[9] and was thrice recrystallized. It consumed a theoretical amount of titer, iodimetrically.

Amperometric titration was carried out at 25° by essentially the same methods as reported by Benesch et al.[10] and Kolthoff et al.[11] However, in the latter, free mercury wave was measured at −0.05 V versus SCE without nitrogen bubbling, except in the titration of SDS-denatured protein.

RESULTS

Comparison of the Titration Methods. β-Amylase and ovalbumin were titrated both argentometrically (Fig. 1) and mercurimetrically (Fig. 2). β-Amylase bound eight mercury atoms per molecule, but in the argentometric titration the current increase at the end point was less sharp and the final straight line showed sixteen atoms per molecule.

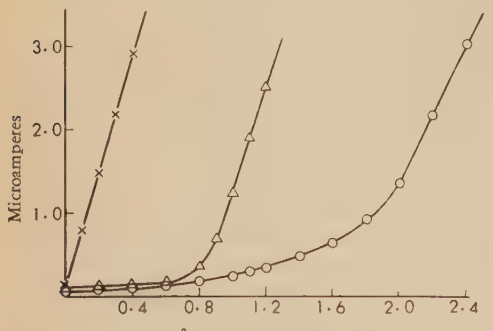

FIG. 1. Amperometric Argentometric Titration Curves of β-Amylase and Ovalbumin

○ 30.3 mg β-amylase
△ 15.1 mg ovalbumin
× reagent line

Titration was carried out in Tris buffer, 0.02 M RNH_2 form and 0.113 M RNH_3^+ form and 0.01 M in KCl, pH 7.4 in a total volume of 15 ml.

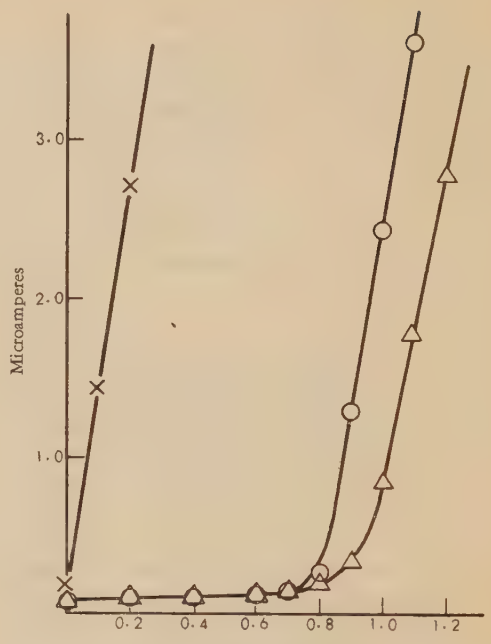

FIG. 2. Amperometric Mercurimentric Titration Curves of β-Amylase and Ovalbumin.

○ 30.3 mg β-amylase
△ 30.2 mg ovalbumin
× reagent line

Titration was carried out in 0.1 M acetate buffer and 0.5 M in KCl, pH 5.6 in a total volume of 20 ml.

Since the maximal number of -SH groups for this enzyme can not exeed ten, the above argentometric data are considered to be too high.

Although the reason for such high argentometric titration results was unclarified, mercurimetric titration was preferred thereafter. No increase in titratable -SH groups was observed after denaturarion with SDS (1000 moles SDS per mol enzyme).

Ovalbumin was found to bind 5.0_2 atoms of silver and 2.9_0 atoms of mercury on the average, after a series of six determinations.

Interrelation between Residual activity and the -SH Content of β-Amylase Treated with o-Iodoso-

7) S. Schwimmer, *Cereal Chem.*, **24**, 167 (1947).
8) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).
9) F. P. Chinard and L. Hellerman, "Methods of Biochemical Analysis" Interscience, 1954, Vol. 1, p. 1.
10) R. E. Benesch et al., *J. Biol. Chem.*, **216**, 663 (1955).
11) I. M. Kolthoff et al., *Anal. Chem.*, **26**, 366 (1954).

benzoate. Oxidation of the enzyme was carried out in 0.1 M phosphate buffer (pH 7.5) at 25°. The reaction proceeded quite slowly in the presence of excess reagent, especially, in the last quarter of activity (Fig. 3). High concentration of the reagent was not feasible because of its limited solubility at this pH. For amperometric titration of the oxidized enzyme, 4 ml aliquot of the reaction mixture as shown in Fig. 3 was cooled after appropriate incubation time, dialysed against cold distilled water for four days to remove the excess reagent and filled up to 10 ml, from which 3 ml portion was taken. The remainder was used for crystallization, reactivation, electrophoresis and other experiments. In Fig. 4 is plotted the residual activity versus the lost –SH groups per molecule. Although the essentiality of –SH groups is demonstrated in Fig. 4, it is impossible to correlate activity to the special –SH groups among the entire eight. Moreover, the interrelation between activity and the remaining –SH groups is not so simple but, it seems as if the first two pairs carry three-fourths and the remaining two pairs one-fourth of total activity. Addition of SDS did not increase the titratable –SH groups for the oxidized enzyme.

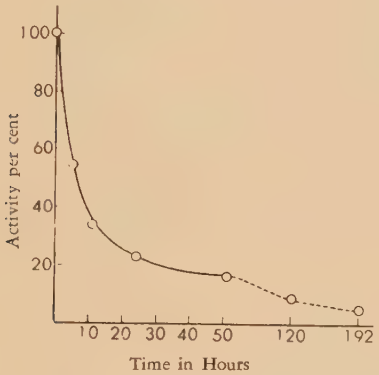

FIG. 3. Time-course of Inactivation of β-Amylase by *o*-Iodosobenzoate.

Two ml portion of β-amylase solution (5.04 %, 8640 units per ml) was incubated with 0.005 M *o*-iodosobenzoate in 0.1M phosphate buffer, pH 7.5, at 25° in a total volume of 4 ml. Activity unit is according to Schwimmer.[7]

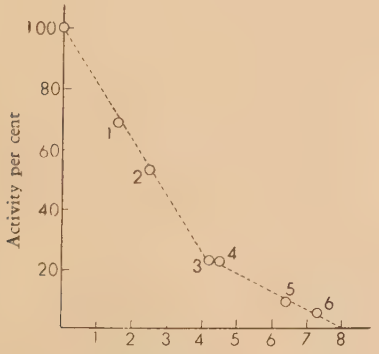

FIG. 4. Relation between Residual Activity and Remaining –SH Groups in β-Amylase Oxidized by *o*-Iodosobenzoate.

The scale of titration corresponding to each point is the same as in Fig. 2. Numerical number indicates that of sample used in other experiments. See text.

Crystallization of Oxidized β-Amylase. Each 5 ml portion of oxidized and partially active enzyme (sample Nos. 1, 2, 3 and 6, shown in Fig. 4, each ca. 1 % solution) was subjected to crystallization in the same manner as in recrystallization of the native enzyme. Some of the crystals obtained are shown in Fig. 5 A, B and C.

These crystals were not the results of preferential crystallization of unoxidized molecules, since when relatively large crystals were collected by centrifugation at a low speed, dialysed against distilled water and assayed for specific activity, they were found to be the same as those contained in the mother solution of crystallization. Only, in the case of sample C in Fig. 5, it was slightly higher (8% of the original activity) than that of the mother solution (6 %).

Thus, the enzyme which has lost half of its original activity can crystallize in the same form as the native one (Fig. 5A).

The reason why such grain-like crystals as shown in Fig. 5B and C were obtained is not clear, but it may be due to the too rapid addition of saturated ammonium sulfate solution in such a small-scale crystallization, since it has often been experienced that the native enzyme crystallizes as a relatively round form

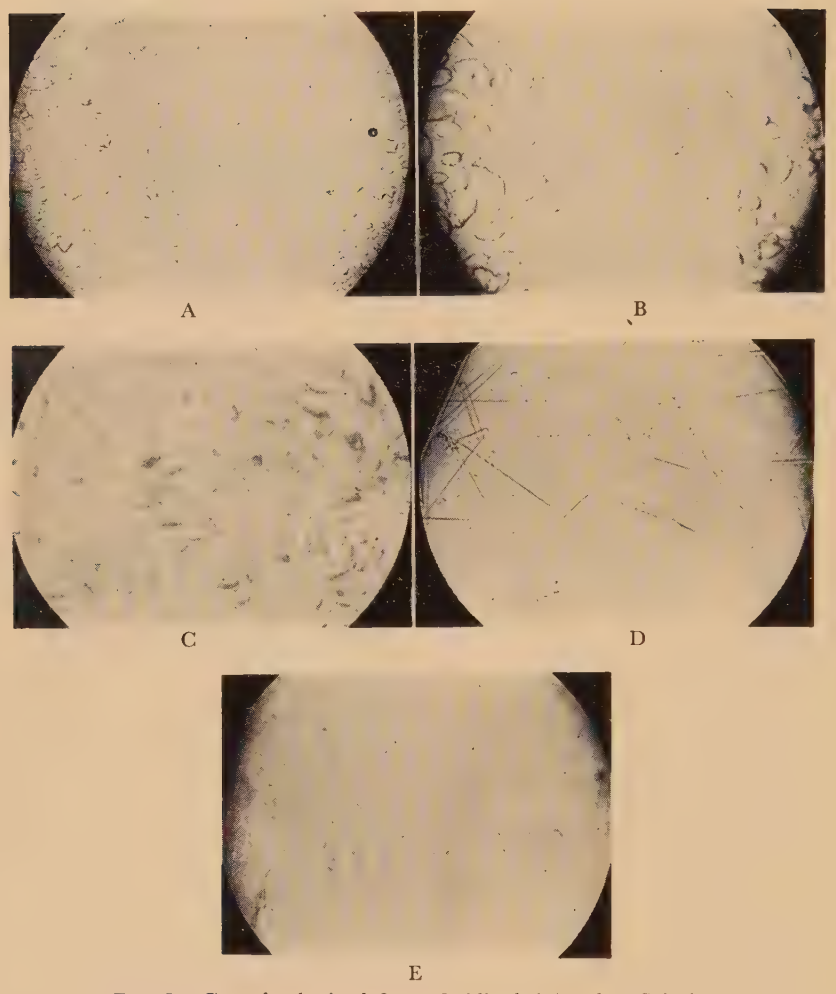

FIG. 5. Crystals obtained from Oxidized β-Amylase Solution

A)	Sample	No. 2 in Fig. 4	(by salting out with ammonium sulfate)	× 50
B)	〃	No. 3 〃	(〃)	×200
C)	〃	No. 6 〃	(〃)	×200
D)	〃	No. 6 〃	(from water by dialysis)	×200
E)	〃	No. 1 in Fig. 8	(〃)	×200

devoid of a sharp edge of the normal crystal and once a grain-like crystal was also observed mixed with a round one.

As oxidation proceeded extensively, the enzyme seemed to become less stable at pH 3.7 and 0.2 saturation of ammonium sulfate. For this reason, crystallization of sample C in Fig. 5 was carried out at pH 3.9, the activity yield being 60 per cent. When the abnormal crystal thus obtained, was dialysed against distilled water for four days, it crystallized from water as a long plate shown in Fig. 5D. The specific activity of this crystal was equal to that of the supernatant and this finding suggests that the oxidation of the enzyme does not proceed by the "all or none" mechanism. If the native enzyme really does exist, it must accumulate in the supernatant, since it does

not crystallize upon desalting.

Reactivation of the Oxidized Enzyme. In order to ascertain whether the enzyme oxidized by *o*-iodosobezoate can be reactivated by the reducing agent, the following two lines of experiment were performed. Sodium thioglycolate was used as the reducing agent, since it had been reported to be more effective than cysteine.[12]

First, a 0.5 ml portion of sample No. 1 in Fig. 4 (activity 69%, 930 units per ml) was incubated with 0.2 M phosphate buffer (pH 7.8) and 0.02 M sodium thioglycolate in a total volume of 1 ml at 25°. After seven minutes and thirty-three minutes of incubation activity was measured, but no change was observed. Second, 5 ml portion of sample No. 4 in Fig. 4 (activity 23%, 319 units per ml) was incubated with 0.1 M phosphate buffer (pH 7.8) and 0.063 M sodium thioglycolate in a total volume of 6.25 ml. No change in activity was observed after fifty minutes, two hours and five hours of incubation. After six hours the solution was transferred into a cellophane sack and was dialysed against distilled water for five days. The dialysate was analysed for both specific activity and the sulfhydryl content, but these were found to remain unchanged. It may be concluded from these results that the reduction of oxidized –SH groups and the restoration of activity are very difficult in sweet potato β-amylase.

Electrophoresis and Sedimentation. Electrophoretic separation was attempted in case of sample No. 5 shown in Fig. 4 (activity 8.5%) mixed with native enzyme in the proportion of 3 : 2, but resulted in failure. (Fig. 6A) Sedimentation was also carried out with 0.8 per cent solution of sample No. 5 in phosphate buffer (pH 7.5, $\Gamma/2=0.1$) at 57,600 rpm and 16.4° by a Spinco Model E Ultracentrifuge.

The value of $S_{20}=8.72s$ was obtained and the finding of Englard et al. was reconfirmed.

Oxidation by Iodine. In contrast to *o*-iodosobenzoate, a minute amount of iodine inactivates β-amylase almost instantaneously, so the diminishing of activity and of –SH groups were also tested for this case. Oxidation was performed in 0.1M acetate buffer (pH 4.8) at room temperature (10°~15°) by adding from 0.002 to 0.01 N iodine solution in a 10-fold concentration of potassium iodide. Although the experimental conditions are not claimed to be the most specific, the stoichiometry is shown in Fig. 7.

At the initial stage the enzyme is inactivated almost instaneously consuming iodine at the

FIG. 6. Electrophoretic Patterns of Oxidized β-Amylase mixed with Native Enzyme.

A) Sample No. 5 in Fig. 4 plus native enzyme (3 : 2), 0.80%
B) " No. 1 in Fig. 8 " (2 : 3), 1.06%
C) " No. 3 in Fig. 8 " (4 : 5), 0.80%

Electrophoresis was carried out at 2° for 10800 seconds in acetate buffer, pH 5.56, $\Gamma/2=0.1$, and at potential gradient of 5.9 V/cm (A and B) or pH 4.83, $\Gamma/2=0.05$ and at 7.2 V/cm (C).

12) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201 (1951).

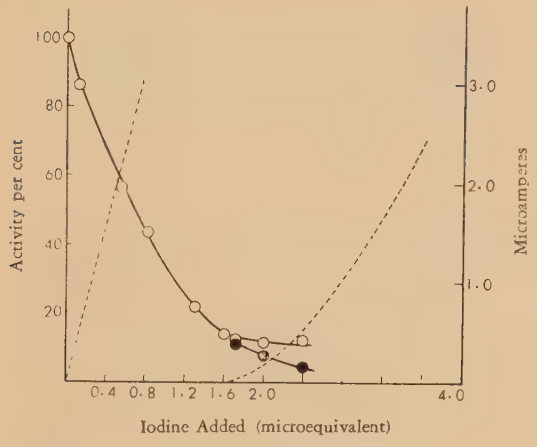

FIG. 7. Stoichiometry in Inactivation of β-Amylase by Iodine.

○ activity immediately after oxidation.
● activity 30 minutes after oxidation.

The amount of iodine expressed is against 10.1 mg β-amylase (1730 units).

Oxidation was carried out in 0.1 M acetate buffer, pH 4.8, at room temperature. Dotted line indicates rapid amperometric iodimetric titration curve of the same amount of β-amylase by 0.002 N iodine in 0.1 M acetate buffer, pH 4.8, and 0.02 M in potassium iodide in a total volume of 15 ml at 25°.

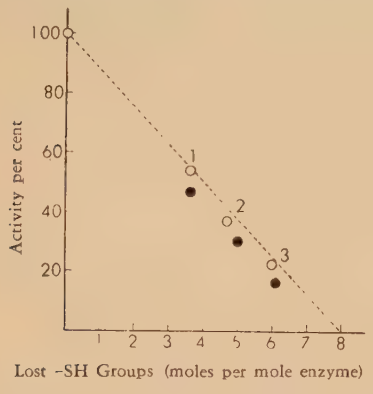

Fig. 8. Relation between Residual Activity and Remaining -SH Groups in β-Amylase Oxidized by Iodine.

○ fresh sample.
● sample stored for 10 days.

Experimental method is the same as in Fig. 4.

rate of 24 to 25 equivalents per mole enzyme, but after 75 to 80 per cent inactivation, the reaction slows down. At this stage free iodine can be observed by amperometric iodimetric titration of the enzyme, in spite of the remaining of activity.

The interrelation between residual activity and remaining -SH groups is shown in Fig. 8. The experimental condition was the same as that of Fig. 4. Namely, to each 2-ml portion of stock enzyme solution was added 5.6, 8.4 and 13.2 microequivalents of iodine at pH 4.8 in a total volume of 4 ml. Thereafter, the reaction mixture was dialysed against distilled water for four days. During dialysis a large amount of thin plate crystal (Fig. 5E) appeared in all the three samples. It was dissolved by adding a minimal amount of 0.5 M acetate buffer (pH 5.6) and the slightly opalescent solution was assayed for -SH content as previously performed. Although the relation was linear and, accordingly, the number of remaining -SH groups in this case was always less as compared with that in *o*-iodosobenzoate-treated enzyme having the same activity, the activity of iodine-treated enzyme was unstable and decreased during storage for about ten days without loss of -SH content.

With sample No. 3 in Fig. 8 the solution was dialysed again and the specific activity of both the precipitate and supernatant and the -SH content of precipitate were tested. They were all found to be the same as in the solution prior to dialysis and evidently the effect of iodine was also not of the "all or none" nature. Electrophoretic separation of samples No. 1 and No. 3 in Fig. 8 from native enzyme was still a failure (Fig. 6-B and C). Moreover, ultraviolet absorption curve of crystal obtained from sample No. 3 in Fig. 8 upon desalting was compared with that of the native enzyme, but no appreciable change could be observed.

DISCUSSION

There have been reported cases where free -SH groups are directly involved in the union of enzyme with the substrate or coenzyme[13], but in hydrolytic enzymes, such as urease, papain and β-amylase, the nature of the essen-

13) P. D. Boyer and H. L. Segal, "Symposium on the Mechanism of Enzyme Action." Johns Hopkins Press, 1954, p. 520.
14) L. Hellerman et al., *J. Biol. Chem.*, **147**, 443 (1943).

tiality is unclarified. Although it is of particular importance that Hellerman[14] had distinguished the reactivity and essentiality of -SH groups in urease, all of the titratable -SH groups in sweet potato β-amylase are essential.

Putnam,[15] on the essentiality of protein groups, describes this as follows:

"When the enzyme is completely inhibited by binding with a single mole of reagent, the essentiality of a certain group or site is demonstrated. On the contrary, when extensive substitution or blocking only diminishes activity, it is possible that the effect is nonspecific."

Because of intermediary number of groups, unequal responsibility of four pairs of -SH groups for activity and the large size of the substrate molecule, it seems difficult to interprete precisely the result shown in Fig. 4. However, it is presumed that the presence of four active centers corresponding to each pairs of -SH groups is scarecely possible and that the essentiality of -SH groups may in this case rather be indirect.

In addition to the intramolecular nature of the oxidation of this enzyme as established by Englard et al., it is highly probable that the results of oxidation either by *o*-iodosobenzoate or by iodine consist of almost equally oxidized molecule and are not of the "all or none" nature. Moreover, there may be some difference of susceptibility towards oxidizing agent in four pairs of -SH groups and the oxidation may occur to certain extent in order and not at random.

The crystallizability of the oxidized enzyme is by no means demonstrative of homogeneity and there must rather exist some microheterogeneity concerning the number of the -SH groups, but the detection seems difficult by sedimentation or usual electrophoretic technique. However, crystallizability offers a criterion of the absence of degradative changes, although the completely inactivated enzyme has not been tested.

The lack of reactivation following oxidation of -SH groups by *o*-iodosobenzoate is exactly the same as reported by Englard et al. The authors have some doubt on the reversible change of activity in the oxidation of this enzyme, since it has never been observed that the enzyme restores its activity by cysteine after oxidation with hydrogen peroxide or cupric ion plus ascorbic acid.[16]

Lastly, the modification of enzyme molecule caused by treatment with iodine is most difficult to interprete. From the equivalence number of iodine consumed per mole enzyme, it is probable that oxidation may proceed beyond the S-S stage, or otherwise groups other than -SH may be attacked, although activity and -SH groups partly remain. The most characteristic feature of iodine-oxidized enzyme is its change in solubility. It behaves like euglobulin even in a state of half inactivation, whereas, on *o*-iodosobenzoate-treated enzyme the same feature can be observed only in more extensive inactivation. Further studies on the reaction condition more specific for -SH groups may be necessary in this case.

The question whether any difference may exist in enzymatic properties between native and oxidized enzymes seems also to be of importance and studies along these lines are in progress.

Acknowledgement. The authors wish to express their sincerest thanks to Prof. S. Funahashi, University of Tokyo, for his encouragement and interest in this work. Thanks are also due to Dr. M. Takanami, National Institute of Animal Health, for the sedimentation analysis.

15) F. W. Putnam, "The Proteins" Academic Press, 1953, Vol. 1, p. 969.

16) M. Ito and M. Abe, *J. Agr. Chim. Soc. Japan*, **28**, 368 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 294~298, 1958]

Rheological Studies of Wheat Flour Dough

Part I. Measurement of Dynamic Visco-elasticity

By Toru Shimizu and Akiyoshi Ichiba

Central Laboratory of Nisshin Flour Milling Co.

Received May 10, 1958

Dynamic rigidity G', dynamic viscosity η' and the relaxation spectrum $L(t)$ of dough under periodic shear stress have been obtained. At first, G' and η' decreased rapidly with rest time, but maintained a constant value after 60 minutes. Values of both G' and η' decreased, and $L(t)$ became flat with the decrease in water absorption and with the temperature. G' increased slightly with the increase in salt content, but η' decreased in a low frequency range.

INTRODUCTION

The rheological behavior of wheat flour dough has been considered to be a more important field of research in cereal chemistry. Schofield and Scott Blair[1] have adopted dough as a typical example of soft materials and have pointed out its intricate mechanical behaviors. And, many investigators have obtained numerous data using practical instruments, for example, such instruments as the Farinograph, Extensograph and Alveograph. Details of these have been reviewed by Bailey[2] and more recently by Greup, Hintzer[3] and Aitken[4].

Recently, Cunningham, Hlynka and Anderson[5,6,7] have devised a relaxometer, and measured the stress relaxation of dough by application of this instrument. The measurement of visco-elasticity may be divided in static and dynamic methods. Stress relaxation is a typical example of the static method. On the other hand, Slater[8] has illustrated a method by which the dynamic property of concentrated flour-water suspension has been measured. In order to observe dynamic visco-elasticity of dough under periodic shear stress, the authors have modified the method described by Nakagawa and Senō[9]. This method is adapted to a low frequency range: for the purpose of obtaining an over-all relaxation spectrum, it is necessary to use it in combination with other methods. But when the bread making process is taken into consideration, rheological behavior in the low frequency range would be especially useful.

EXPERIMENTAL

1. Apparatus. A sketch of the apparatus is given in Figure 1. Dough ⑪ is set in a cylindrical cup ⑤ (radius $r_1=2.84$ cm) which is supported by a stand ⑦ and put in a thermoregulated water-bath ⑥. A cylindrical bob ④ (radius $r_2=2.00$ cm), whose end is cone-shaped, is inserted into the center of the dough. The bob is suspended by a piano wire ③ and a driving device ① gives an oscillation $\theta=\theta_0 \sin \omega t$ to the top of the wire ②. The bob, which is inserted in the visco-elastic body, shows a different oscillation $\theta=A\sin(\omega t-\phi)$. To measure the amplitude ratio $p=$

1) R. K. Schofield and G. W. Scott Blair, *Proc. Roy. Soc.* **A. 138**, 707 (1932) ; **A. 139**, 557 (1933) ; **A. 141**, 72 (1933) ; **A.160**, 87 (1937).
2) C. H. Bailey, *Wheat Studies of the Food Research Institute*, **16**, 242 (1940).
3) D. H. Greup and H. M. R. Hintzer, "Foodstuffs" ed. by Scott Blair, North Holland Pub. (1953).
4) T. R. Aitken, *A. A. C. C. Trans.*, **12**, 157 (1954).
5) J. R. Cunningham and I. Hlynka, and J. A. Anderson, *Can. J. Technol.*, **31**, 98 (1953).
6) J. R. Cunningham and I. Hlynka, *J. Appl. Phys.*, **25**, 1075 (1954).
7) I. Hlynka and J. A. Anderson, *Agr. & Food Chem.*, **5**, 56 (1957).
8) L. E. Slater, *Food Eng.*, **26**, May 74 (1954).
9) T. Nakagawa & M. Senō, *Bull. Chem. Soc. Japan*, **29**, 471 (1956).

A/θ_0 and the phase difference ϕ, the optical Lissajous' method was adopted.

Small galvanometer mirrors M_1 and M_2 are fixed to the top of the piano wire, and to the bob. A convex lens L is placed in front of M_1 and M_2. Light emitted from the source at ⑧ is made parallel by L, is reflected by M_1 and M_2, and is then converged by L. A strip mirror M_3 is placed at an angle of 45° to the beam of light in the camera ⑨. M_3 is connected to the oscillation mechanism and a photographic paper ⑩ is placed at the focus of L. Two Lissajous' figures, the one a linear line Ⓛ and the other an elliptic curve Ⓔ from two oscillations whose directions are at right angles to each other, are obtained. A typical Lissajous' figure is shown in Figure 2.

2. Calculations. Equation of motion of the bob is expressed as follows:

$$I\ddot{\theta} = -k_0\theta - R\dot{\theta} - K\theta \qquad (1)$$

where, I Moment of inertia of the bob.
k_0 Torison constant of the piano wire.

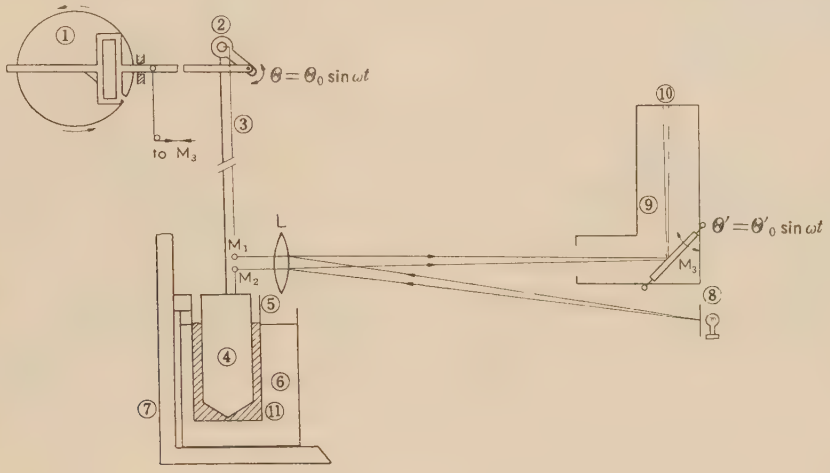

FIG. 1. Schematic diagram of the instrument.

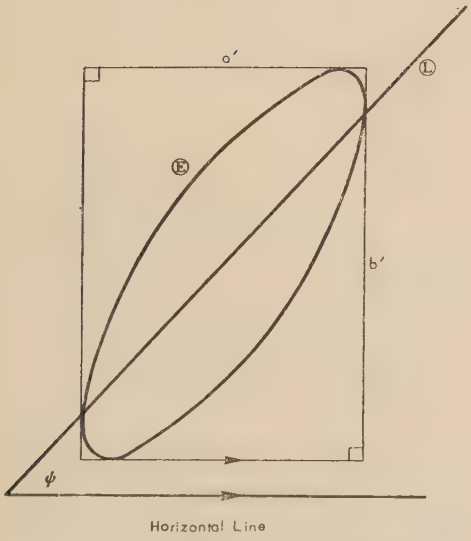

FIG. 2. Typical Lissajous' figures.

R Resistance of dough.
K Elastance of dough.

Nakagawa and Senō[9] have indicated a relationship between the data obtained from the Lissajous' figure and the solution of Equation (1), which is as follows:

$$\left.\begin{aligned} \sin\phi &= \frac{4\,(S)}{\pi\,[S]} \\ p &= \frac{b'}{a'}\tan\phi \end{aligned}\right\} \qquad (2)$$

$$\left.\begin{aligned} K &= k_0\left(\frac{\cos\phi}{p} - 1\right) + \frac{4\pi^2 I}{T_f^2} \\ R &= \frac{k_0 \cdot T_F \cdot \sin\phi}{2\pi p} \end{aligned}\right\} \qquad (3)$$

$$\left.\begin{aligned} G' &= \frac{K}{4\pi l}\left(\frac{1}{r_1^2} - \frac{1}{r_2^2}\right) \\ \eta' &= \frac{R}{4\pi l}\left(\frac{1}{r_1^2} - \frac{1}{r_2^2}\right) \end{aligned}\right\} \qquad (4)$$

where, G' Dynamic rigidity of dough.
η' Dynamic viscosity of dough.
$T_F\,(=2\pi/\omega)$ Period of oscillation.
(S) Area of elliptic curve.
$[S]\ (=a'b')$ Area of the rectangle whose one side runs

parallel with the horizontal line and each side is tangent to the curve (See Fig. 2).
ψ Angle between L and the horizontal line.
l Depth of dough.

The real and imaginary part G', G'' of complex dynamic modulus G^* can be expressed by the relaxation spectrum $L(t)$ as follows:

$$\left.\begin{aligned} G' &= \int_{-\infty}^{+\infty} \frac{\omega^2\tau^2}{1+\omega^2\tau^2} L(t)d\log\tau \\ \eta' &= \int_{-\infty}^{+\infty} \frac{\omega\tau}{1+\omega^2\tau^2} L(t)d\log\tau \end{aligned}\right\} \quad (5)$$

Schwarzl[10] has indicated a method by which $L(t)$ is obtained approximately as follows:

$$\left.\begin{aligned} L(t) &= \frac{2}{\pi}[G''(\omega)]_{\omega=\tau^{-1}} \\ G''_{(\omega)} &= \omega\eta'_{(\omega)} \end{aligned}\right\} \quad (6)$$

The following data were obtained with the aid of of Equations (2), (3), (4), (6).

3. Material and Experimental Procedure. A long patent flour obtained from Canadian Manitoba Northern # 3 wheat was used in this study. The flour contained 0.41 % ash and 11.3 % crude protein on a 14 % moisture basis. Dough was prepared by mixing the flour in Kenwood mixer for 1.5 min.. A hundred grams of dough was rounded by a Brabender Extensograph rounder 20 times and set in the cup. After a constant rest period, a forced oscillation (Amplitude, 0.062 radian) was given to the top of the piano wire. The diameter of the wire was 2.4 mm and the torsion constant was 3.65×10^6 dyne.cm.

RESULTS AND DISCUSSION

1. Changes of G', η' with Rest Time. Results are shown in Figure 3. The experimental conditions were;

Temperature 28°C, Water absorption 63 %, 66 %, Period 2.7 sec..

At first, both G' and η' of dough decreased rapidly, but maintained a constant value after about 60 minutes. It is thought that these results are related to the structural relaxation of dough which has been reviewed by Hlynka and Anderson[7]. From these results, a rest time of 60 minutes was selected for the following experiments.

2. Changes of Rheological Properties at different Water Absorptions. G', η' and $L(t)$ of 63 % and 66 % water absorption are shown in Figure 4. Data represent the function of period. The

10) F. Schwarzl, *Inter. Cong. Rheology*, **1953**, 197.

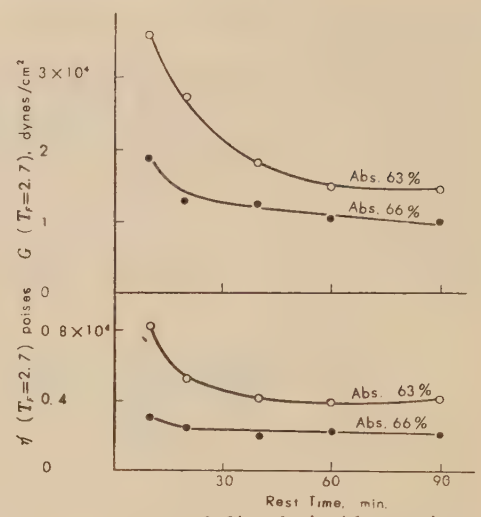

FIG. 3. Changes of G' and η' with rest time.

experimental conditions were;

Temperature 28°C, Rest time 60 min.,

Period 1.7, 2.7, 10.1, 23.5 sec..

G' and η' decreased and $L(t)$ became flat with the increase in water absorption. These results correspond to those of Halton and Scott

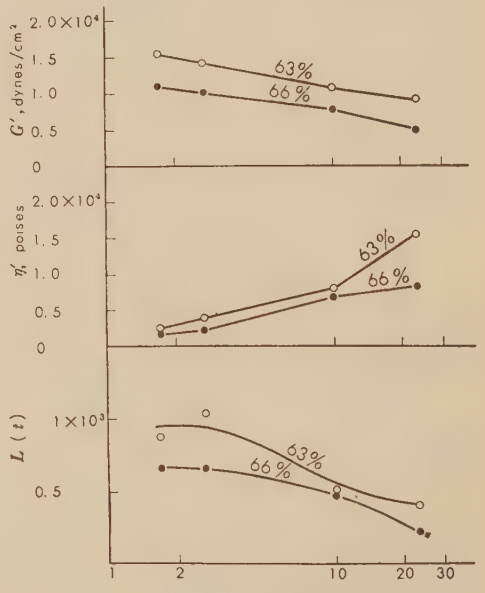

FIG. 4. Changes of G', η' and $L(t)$ at different water absorptions.

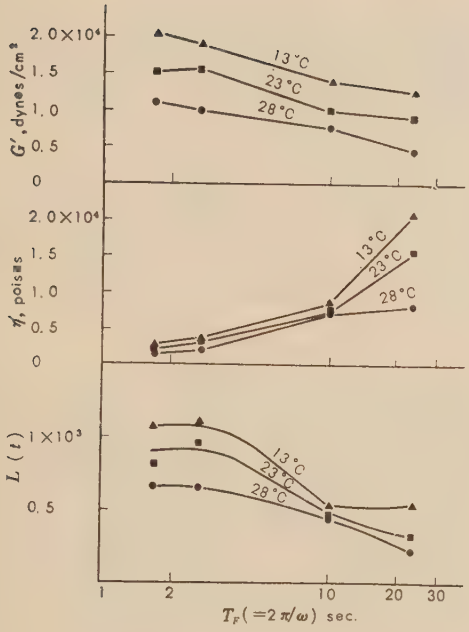

FIG. 5. Changes of G', η' and $L(t)$ at different temperatures.

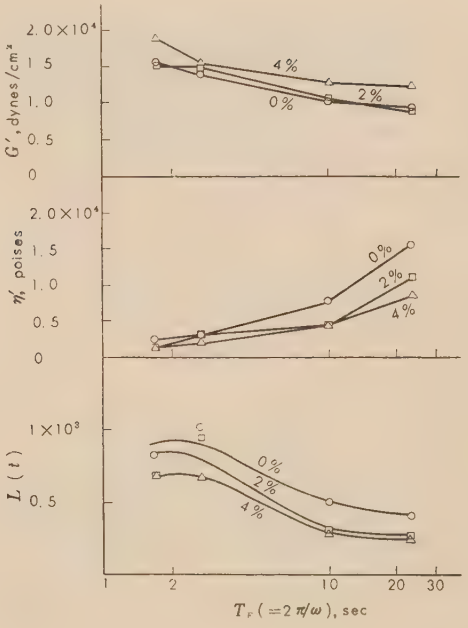

FIG. 7. Changes of G', η' and $L(t)$ at different salt contents.

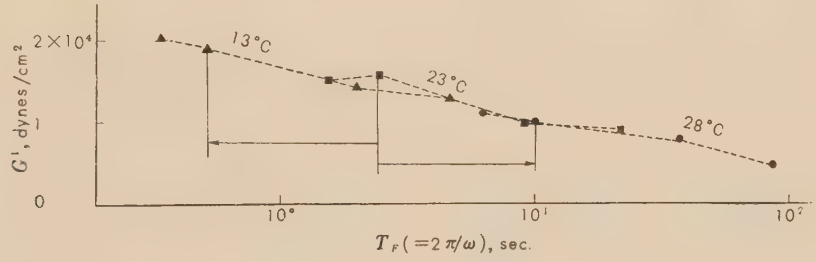

FIG. 6. Shift of Figure 5.

Blair[11] and Cunningham and Hlynka[6] obtained in static experiments.

3. Changes of Rheological Properties at Different Temperatures. G', η' and $L(t)$ at 13°C, 23°C and 28°C are shown in Figure 5. Water absorption was 66% and other experimental conditions were the same as in the former experiment.

G' and η' decreased and $L(t)$ became flat with the increase in temperature. When the $G'-\log. T_F$ relationship at 13°C was shifted to the left along the time axis and the relationship at 28°C to the right, they formed part of a master curve as that in Figure 6. From this fact, it is suggested that flour dough is approximately thermorheologically simple as many high polymers and therefore the data in the low temperature range can be used instead of that in the high frequency range.

4. Changes of Rheological Properties at Different Salt Content. G', η' and $L(t)$ at zero, 2 and 4% salt content to flour are shown in Figure 7. Water absorption was 63% and other experimental conditions were the same as these

11) P. Halton and G. W. Scott Blair, *Cereal Chem.*, **14**, 201 (1937).

in the former experiment.

G' increased slightly with the increase in salt content, but η' decreased in the low frequency range. As it is anticipated that inorganic ions have a stroug influence on the rheological properties of dough, this problem will be the subject of future investigation.

These results lead to the following comments.
1) If a four parameter mechanical model can be assumed for dough, the relation between static and dynamic rigidity may readily be expressed as:

$$G'=G_1+G_2\frac{\omega^2\tau_2^2}{1+\omega^2\tau_2^2} \qquad (\tau_2=\eta_2/G_2) \qquad (7)$$

As elasticity, which has been obtained by Halton and Scott Blair[10], is a mixture of instantaneous rigidity G_1 and retarded rigidity G_2, the order of magnitude G' of the present experiments is the same as that of the former data.

2) Accuracy of the approximation by which relaxation spectrum is obtained, is in the same order magnitude as that of Cunningham and Hlynka[6]. Moreover, this approximation method is very simple.
3) In contrast to data obtained from dough testing machines, the authors can obtain the data in c.g.s. units by this method. It is probable that these data are comparable with those of other materials other than wheat flour dough.

Acknowledgement. The authors wish to express their sincere thanks to Dr. T. Nakagawa of Tokyo University for his constant guidance, and to Dr. I. Hlynka of the Grain Research Laboratory, Winnipeg, Canada, for his kind advice. They also wish to thank Mr. H. Shoda, president of Nisshin Flour Milling Co., and Mr. T. Yasui, director in charge of this laboratory, for his encouragement in this work.

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Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria

Part IX. Formation of Ketopentoses by Lactic Acid Bacteria*

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Received May 22, 1958

The formation of ketopentoses from aldopentoses was demonstrated by six strains of heterofermentative lactic acid bacteria (heterofermenters). The dried bacterial cells harvested on malt extract and their cell-free extracts were found to reveal isomerization of D-xylose or L-arabinose to corresponding ketopentoses in the presence of borate, while any formation of ketopentose was never observed with the enzyme preparations of homofermenters except *L. xylosus* and *Pc. lindneri*. The ketopentoses were isolated by a Dowex-1 borate column, and identified by paper-chromatography. Results obtained were as follows: xylulose was formed from xylose by six strains of heterofermenters (*L. fermentum, Leuc. mesenteroides, L. brevis, L. buchneri, L. gayonii* and *L. fermenti*) and by *L. xylosus*. Ribulose was obtained from arabinose by *L. brevis, L. pentoaceticus, L. gayonii, L. buchneri* and *Pc. linderi*.

INTRODUCTION

Fermentation of glucose by heterofermenters is characterized by the formation of lactic acid, ethanol and carbon dioxide at the equimolar proportion. On their pentose fermentation, both arabinose and xylose are converted into acetic and lactic acids, and reports on isotopic analysis of the decomposition products of 1-C^{14} labeled arabinose,[1] xylose[2,3] or ribose[4] by *L. pentoaceticus* or *L. pentosus* have already been published. Lampen and Peterjohn[5] have pointed out the specificity of pentose fermentation by *L. pentosus*.

It was first described by Cohen[6] that the formation of ribulose from D-arabinose takes place by the extracts of *Escherichia coli* mutant, according to isomerization of pentose. Similarly, arabinose isomerase was also found in cell-free extracts of *L. pentosus*[7] and *Aerobacter aerogenes*[8] grown on media containing arabinose. Xylose isomerase, which catalyzes the interconversion of D-xylose and D-xylulose, has been demonstrated in the extracts of cells of *Pseudomonas hydrophyla*,[9] *L. pentosus*[10] and *Pasteurella pestis*[11] harvested from xylose media. The arabinose and xylose isomerases have been further studied with extracts of *L. pentosus*, a homofermenter, though the activities of these enzymes of heterofermenters have

* Presented in part before the 141th regular meeting of Kansai Branch of the Agricultural Chemical Society of Japan (June, 1957).

The following abbreviations applied here indicate: trichloracetic acid: TCA; tris-(hydroxymethyl)-aminomethane: Tris and adenosine triphosphate: ATP.

1) D. A. Rapport, H. A. Barker and W. Z. Hassid, *Arch. Biochem.*, **31**, 326 (1951).
2) H. Gest and J. O. Lampen, *J. Biol. Chem.*, **194**, 555 (1952).
3) J. O. Lampen and H. Gest, *J. Bacteriol.*, **61**, 97 (1951).
4) I. A. Bernstein, *J. Biol. Chem.*, **205**, 309 (1953).
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8) F. J. Simpson and W. A. Wood, *J. Am. Chem. Soc.*, **78**, 5452 (1956).
9) R. M. Hochster and R. W. Watson, *Arch. Biochem.*, **48**, 120 (1954); *Canad. J. Microbiol.*, **1**, 346 (1955).
10) S. Mitsuhashi and J. O. Lampen, *J. Biol. Chem.*, **204**, 1011 (1953).
11) M. W. Slein, *J. Am. Chem. Soc.*, **77**, 1663 (1955).

yet not been investigated.

In this paper, xylose- and arabinose-isomerases in heterofermenters are described. The activities of these enzymes with homofermenters are not pointed out except those of *L. xylosus* and *Pc. lindneri*, so that some observations are instituted to discuss why activities of pentose isomerases are different among the types and species of lactic acid bacteria.

METHODS

Microorganisms. Six strains of heterofermenters (*L. fermentum* β, *Leuc. mesenteroides* α, *L. brevis* ATCC 8281, *L. buchneri*, *L. gayonii* ATCC 8289, and *L. fermenti* ATCC 9338) and seven strains of homofermenters (*L. delbrückii*, *L. casei*, *L. plantarum*, *L. bulgaricus*, *L. arabinosus* ATCC 8044, *L. xylosus* and *Pc. lindneri*) were used. These bacteria were cultured in malt extract from 24 to 40 hours at 37°C, harvested, washed twice with distilled water and dried *in vaccuo*. From the dried cells thus obtained, crude cell-free extracts were prepared by grinding with alumina.

Chemicals. Xylose and arabinose employed were Merck products. Barium salt of ATP was prepared from rabbit muscle[12]. Xylulose and ribulose were prepared by epimerization in pyridine under a reflux condenser[13,14].

Enzymatic Assay. The reaction system was composed of 15 mg dried cells suspended in 0.5 ml of distilled water (or 1.0 ml of extracts from dried cells), 50 μM of phosphate buffer at pH 7.0, 10 μM of $MgCl_2$ and 20 μM of pentose. The total volume was adjusted to 2.0 ml with distilled water. A series of parallel experiments were carried out with the reaction systems adding 20 μM of sodium tetraborate. The reaction mixture was incubated at 37°C from one to twenty-four hours. The reaction was stopped by adding 0.2 ml of 20% TCA, washed twice by 1.0ml of 5% TCA and centrifuged. Aliquots were then analyzed.

Ketopentose was separated by the method of ion-exchange chromatography[15]. A Dowex-1 (borate form, 100–300 mesh) column (1.5×20 cm or 1.5×14 cm) was used and the sugars eluted with 0.02 and 0.04 M sodium tetraborate. The flow rate was 0.5 ml per minute and each 25 ml of eluate fraction was collected.

Determinations. Reducing suggars were determined by the Folin-Malmros method[16]. Ketopentose was estimated by the cysteine-carbazole reaction.[17] The red purple color was developed at 20°C, and the colors were read after 20 minutes in case of ribulose and 120 minutes with xylulose[18,19]. For the detection of ketopentose, a resorcinol reaction by Kulka[20] and orcinol reaction of Drury[21] were adopted by heating for 40 minutes. Oxidation of pentose by bromine was carried out with 0.5 ml of bromine saturated water and 30 mg of $BaCO_3$ at 25°C for 30 minutes. In this condition, 2000 μM of xylose must be oxidized, so that the residual amount is made up to approximately 2.8 μM.

For identification of ketopentose, paperchromatographic procedures were made under several solvent systems, and the spots were developed by spraying either anilline hydrogene pathalate or orcinol-TCA. Protein was determined by Lowry's method[22] and phosphate by the method of Fiske and SubbaRow[23].

Spectrophotometric measurements were made with a Shimadzu spectrophotometer, Model QB 50.

RESULTS

1. Detection of Ketopentoses.

Formation of ketopentoses from xylose, arabinose and rhamnose were examined. In general, these pentoses are supposed to be metabolized into such final products as lactic and acetic acids by fermentation. Cohen[6] has firstly observed the effect of borate to generate ketopentose enzymatically by *E. coli* mutant, therefore borate was added as the trapping agent for ketopentose throughout the present experiments.

When D-xylose or L-arabinose were incubated with the cell-free extracts or dried cells, in the presence of borate, the cysteine-carbazole test was positive. Some typical absorption spectra of generated products by both cysteine-

12) P. S. Krishnan and W. L. Nelson, *Arch. Biochem.*, **19**, 65 (1948).
13) O. T. Schmidt and R. Treiber, *Ber.*, **66**, 1765 (1933).
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22) O. H. Lowry, N. J. Rosenbrough, A. L. Forr and R. T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
23) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

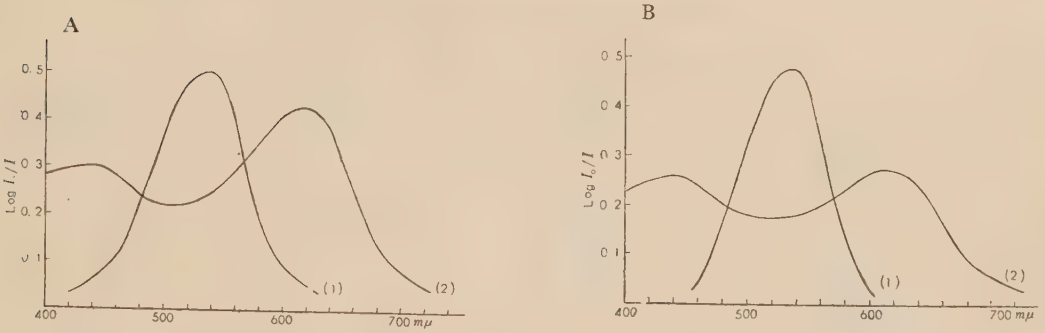


FIG. 1. Spectra of Ketopentose.

A. Formed from xylose by *L. fermentum*. (1) Cysteine-carbazole reaction.
B. Formed from arabinose by *L. brevis*. (2) Resorcinol reaction.

carbazole and resorcinol reactions are given in Fig. 1. This figure shows that the absorption maxima are 540 mμ in the former and 620 mμ in the latter reaction. The same results were obtained with all of the reaction-products formed from xylose and arabinose. When boiled bacterial preparations of various species were used, cysteine-carbazole reaction was always negative with xylose, arabinose and rhamnose. Thus from Fig. 1, it seems possible to consider that the reactive substance of both cysteine-carbazole and of resorcinol reactions is ketopentose.

The activities of isomerazation of pentoses by both hetero- and homofermenters are summerized in Tables I and II. With all homofermenters except *L. xylosus*, no activity was observed at one hour's incubation. Moreover, the activities of cell-free extracts prepared from dried cells are shown in Table III.

TABLE I FORMATION OF KETOPENTOSE
(Reaction time, 1 hour)

		Ketopentose formed (μM)			
		xylose		arabinose	
	Addition of borate	−	+	−	+
Heterofermenter	*L. fermentum*	0.3	2.4	0	0
	Leuc. mesenteroides	0.9	2.6	0.3	0.3
	L. brevis	0.3	0.4	2.4	5.9
	L. pentoaceticus	0.07	0.07	2.6	6.3
	L. gayonii	0.1	0.2	0.3	2.2
	L. buchneri	0.7	0.7	1.9	4.2
	L. fermenti	0.3	0.4	0.2	0.2
Homo-	*L. xylosus*	1.9	23.2	0	0
	Pc. lindneri	0.3	0.4	2.6	5.9

From the results of the above experiments, it is shown that ketopentoses are actually formed and accumulated remarkably in the presence of borate, and the ability of isomerization of these pentoses can be pointed out only by heterofermenters, and not by homofermenters except two of these, *L. xylosus* and *Pc. lindneri*.

2. Effect of Borate.

The experimental results shown in Table I and II, are obtained only in the case with 20 μM of borate. Fig. 2 represents three such cases as xylose isomerase of *Leuc. mesenteroides* and *L. xylosus*, and arabinose isomerase of *L. brevis*. In these experiments, the initial concentration of pentose was always 20 μM, while concentrations of borate were changed from 0 to 50 μM. At the concentration of 20 or 30 μM, borate reveals acceleration on ketopentose formation, but it shows an inhibitory effect at higher concentrations over 30 μM.

3. Fluctuation of Ketopentose Formation by Reaction Time.

Relation between the amount of ketopentose fromation from xylose and the reaction time required by *Leuc. mesenteroides* is shown in Table IV. Both bromine-stable pentose and ketopentose show an increase according to reaction time. Each aliquot of reaction mix-

TABLE II FORMATION OF KETOPENTOSE
(Reaction time, 24 hours)

		Ketopentose formed (μM)							
		xylose			arabinose			rhamnose	
Addition of borate		−	+	%*	−	+	%*	−	+
Hetero-fermenter	*L. fermentum*	0	7.5	37.5	0	0	0	0	0
	Leuc. mesenteroides	0.1	7.2	36.0	0.8	1.2	—	0	0
	L. brevis	3.6	14.6	73.0	3.6	14.6	73.0	0	0
	L. gayonii	1.8	15.9	79.5	2.2	9.2	46.0	0	0
	L. buchneri	2.3	3.8	19.1	2.3	11.0	55.0	0	0
	L. fermenti	1.7	2.1	10.7	0.2	0.2	—	0	0.1
Homo-fermentar	*L. xylosus*	0.1	9.0	44.8	0.8	1.1	—	0	0.03
	Pc. lindneri	1.1	1.2	—	0.4	16.4	81.4	0.8	0.8
	L. delbrückii	0.2	0.3	—	0.3	0.1	—	0.3	0.1
	L. plantarum	0.2	0.1	—	0.5	0.5	—	0.1	0.1
	L. arabinosus	0.3	0.1	—	0.9	2.0	—	0.05	0.06
	L. casei	0.07	0	—	0	0.5	—	1.2	1.0
	L. acidophilus	0.07	0.07	—	0	0.1	—	0.07	1.7
	L. bulgaricus	0.07	0.07	—	0.1	0.1	—	0.08	0.1

* Per cent of isomerization.

TABLE III PENTOSE ISOMERASE ACTIVITIES OF CELL-FREE EXTRACTS

Cells	Extractant		Protein (mg/ml)	Substrate	Pentose isomerase activity (μg ketopentose/mg protein)	
					without borate	with borate
L. fermentum	Water		3.65	xylose	28.1	27.0
	0.05 M	phosphate (pH 7.4)	2.5	〃	40.5	41.7
	0.05 M	Tris (pH 7.4)	2.25	〃	46.3	37.4
	0.05 M	$NaHCO_3$	2.6	〃	38.5	39.4
*L. xylosus**	Water		2.4	xylose	528	1852
	0.05 M	phosphate	2.3	〃	435	2090
	0.05 M	Tris	2.92	〃	418	1564
	0.05 M	$NaHCO_3$	3.62	〃	351	1522
L. gayonii	Water		1.51	arabinose	54.7	60.2
	0.05 M	$NaHCO_3$	2.15	〃	80.2	83.4

150 mg of dried cells was grinded with 300 mg of alumina in the chilled mortar, extracted with 5 ml of extractant. Enzymatic reaction was carried out with Tris buffer (pH 7.4) for 2 hours at 37°C.

* 200 mg of dried cells was used.

tures showed two absorption maxima at 540 and 670 mμ, after bromine oxidation by the orcinol reaction. Ratio of optical densities of the two peaks were 0.37–0.49 and, these evidences thus suggest the reaction product to be xylulose.

With several active strains listed in Table II, formations of ketopentose from xylose and arabinose can also be determined. One-tenth ml of incubation mixture was removed at requisite reaction times and added 0.9 ml of

TABLE IV
FORMATION OF BROMINE STABLE PENTOSE*
(*Leuc. mesenteroides*, xylose isomerase)

Incubation time (min.)	20	40	60	90	120
Br_2-stable pentose (μM)	8.8	11.3	12.6	12.0	11.6
Ketopentose (μM)	6.7	11.4	13.7	13.2	12.9

* 20 μM of borate added.

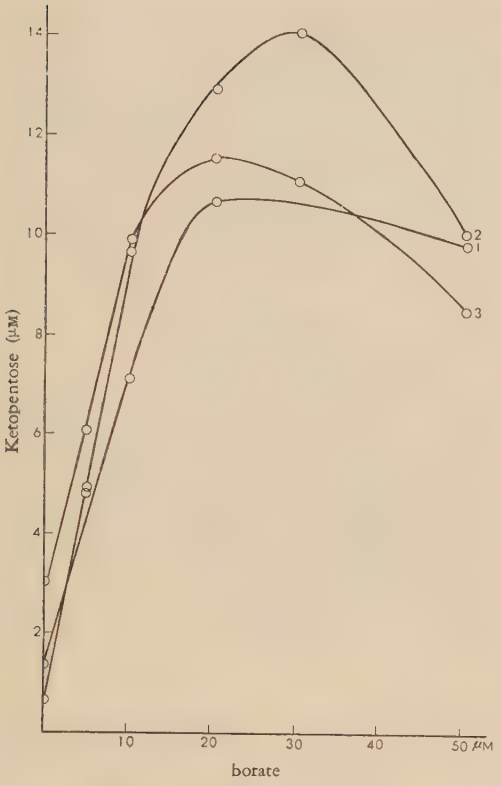


FIG. 2. Effect of Borate.
1. xylose, *Leuc. mesenteroides*,
2. xylose, *L. xylosus*,
3. arabinose, *L. brevis*.

0.1 N HCl. Ketopentose was determined without removing turbidity. As shown in Fig. 3, activity of isomerization of xylose and arabinose seemed to reveal each characteristic property which may be helpful for the classification of these lactic acid bacteria.

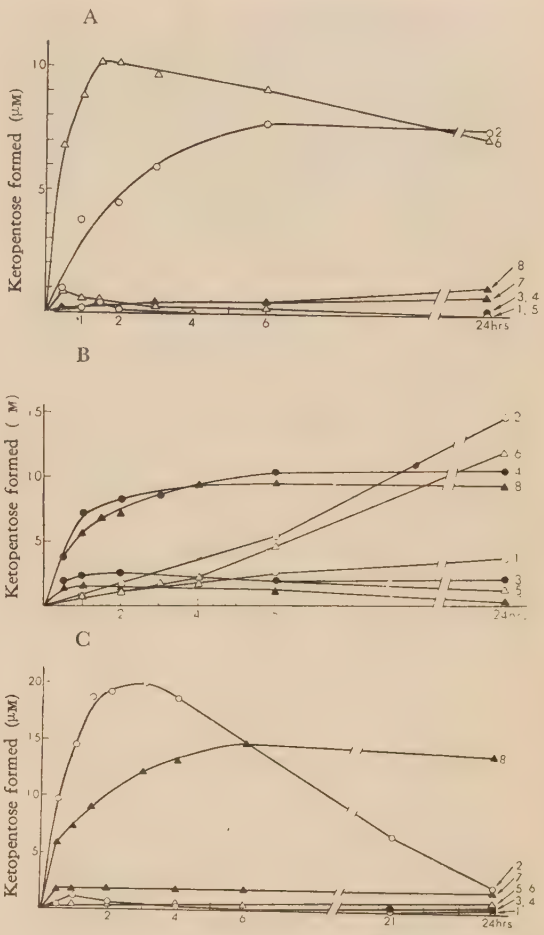


FIG. 3. Fluctuation of Ketopentose Formation.

	cells	xylose	X+borate	arabinose	A+borate
A	*L. fermentum*	1	2	3	4
	Leuc. mesenteroides	5	6	7	8
B	*L. brevis*	1	2	3	4
	L. gayonii	5	6	7	8
C	*L. xylosus*	1	2	3	4
	Pc. lindneri	5	6	7	8

TABLE V EFFECT OF BUFFER

Cells	Pentose	Reaction time (hr.)	Ketopentose formed (μM)		
			Phosphate	Tris	Borate*
L. fermentum	xylose	2	9.2	5.8	9.8
Leuc. mesenteroides	〃	2	2.4	1.2	1.3
L. xylosus	〃	2	8.9	9.1	5.5
L. gayonii	〃	3	1.4	1.7	3.5
	arabinose	2	15.0	14.6	6.4
Pc. lindneri	〃	2	15.2	15.3	6.2

* Addition of borate ommited.

4. Effects of Phosphate and ATP.

A series of experiments were carried out with each 50 μM concentration of phosphate, Tris and borate buffers solutions of pH 7.4 to examine the effects of these buffer solutions on ketopentose formation. As shown in Table V, inorganic phosphate had no effect when compared with Tris buffer, but borate revealed a rather inhibitory effect on ketopentose formation, and any phosphorylated compound of pentose was never obtained in course of these experiments. But, if 150μM of ATP (Na salt) was added to the reaction mixture (total volume, 30 ml) containing 300 μM of pentose, 150 μM of $MgCl_2$, 400 μM of Tris buffer of pH 7.4, 150 μM of sodium borate and 75 mg of dried bacterial cells, phosphorylation of pentose was recognized. Parallel experiments were carried out with phosphate buffer without the addition of ATP. The yields of barium-soluble and alcohol-insoluble fraction are shown in Tables VI and VII. After removing barium with Na_2SO_4 from this fraction, orcinol and cysteine-carbazole reactions were found to be positive and alkali-labile and acid-stable phosphates were also found. Futhermore, it was observed that the alkali-stable pentose was found to be about 81 to 87 per cent of total pentose, so it seems to be presumable that each fraction contains aldopentose- and ketopentose-5-phosphates and triosephosphate, (glyceraldehyde-3-phosphate).

5. Identification of Ketopentoses.

As seen in Tables VI and VII, phosphorylation of pentose does not occur in the absence of ATP. The semi-marco scale experiments were carried out with heterofermenters (xylose isomerization by *L. fermentum* and *Leuc. mesenteroides*, and arabinose isomerization by *L. gayonii*) and homofermenters (xylose isomerization by *L. xylosus* and arabinose isomerization by *Pc. lindneri*) in which the reaction

TABLE VI EFFECT OF ATP

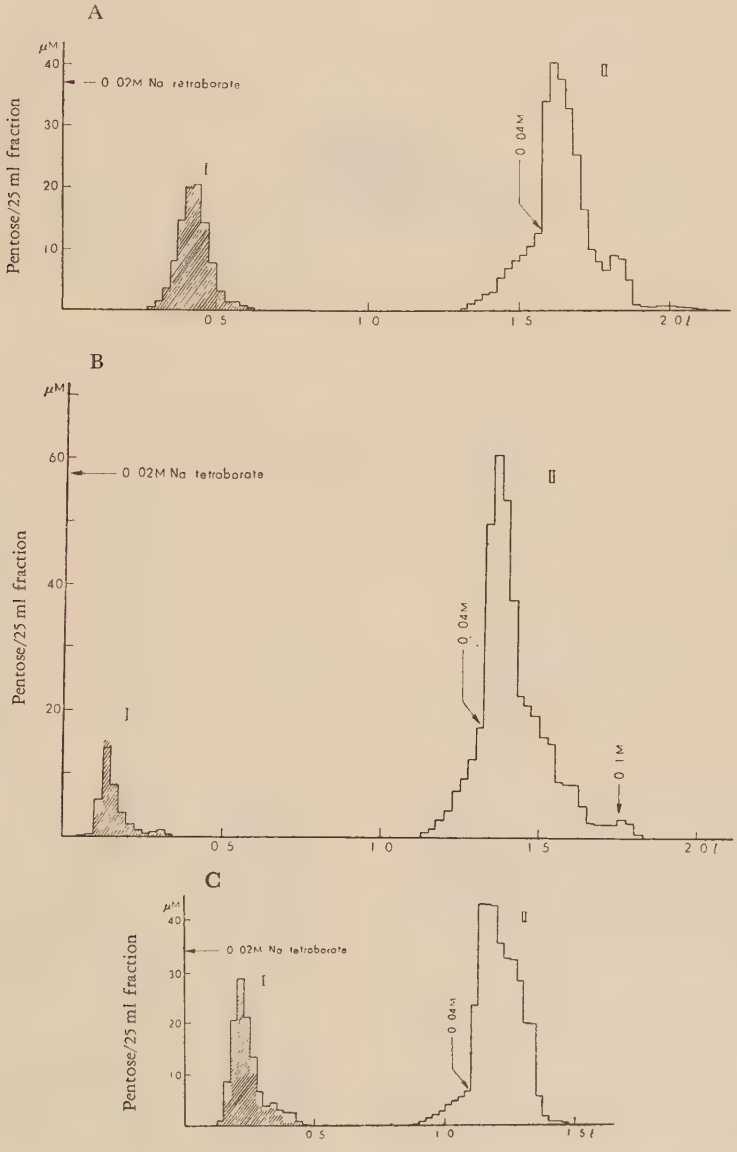
Organism	Substrate	Buffer	ATP added (μM)	Free ketopentose (μM)	Barium soluble EtOH insoluble fraction (mg)
L. fermentum	xylose	Phosphate	0	30.8	3.6
		Tris	150	17.4	28.1
Leuc. mesenteroides	xylose	Phosphate	0	38.5	2.5
		Tris	100	30.6	19.6
L. gayonii	arabinose	Phosphate	0	92.5	4.8
		Tris	150	84.5	30.5

TABLE VII ANALYSIS OF BARIUM SOLUBLE-ALCOHOL INSOLUBLE FRACTIONS

	Without ATP			With ATP		
	1	2	3	1	2	3
Substrate	xylose	xylose	arabinose	xylose	xylose	arabinose
Barium-ppt (mg)	3.6	2.5	4.8	28.1	19.6	30.5
Pentose (μM)	0.7	0.47	0.61	25.2	12.6	26.1
Ketopentose phosphate (μM)	0.18	0.51	0.4	1.43	1.7	7.32
Alkali-stable pentose (μM)	—	—	—	21.8	10.2	21.1
%				86.5	81.0	81.0
Inorganic phosphate (μM)	0	0	0	2.16	1.51	1.91
Organic P (μM)	2.4	2.7	2.5	35.7	15.0	31.7
True acid labile P (μM)	1.26	0.45	1.3	2.31	0.58	2.15
True alkali labile P (μM)	2.26	1.26	1.26	25.1	9.64	18.2
Bial's reaction $D_{549} : D_{670}$	—	—	—	0.18	0.19	0.19

1. *L. fermentum*, 2. *Leuc. mesenteroides*, 3. *L. gayonii*.

mixture of 30 ml of the total volume, was composed of 150 mg of dried bacterial cells, 600 mμ of pentose, 300 mμ of $MgCl_2$, 750 μM of phosphate buffer of pH 7.4 and 300 μM of sodium tetraborate. After incubation for 2 hours at 37°C, the incubation mixture was deproteinized by heating in a boiling-water bath for 5 minutes and then centrifuged. The supernatant was chromatographed on a Dowex-1 borate column, and the fractions were eluted with 0.02 M sodium tetraborate. Consecutive fractions were collected, passed through Amberlite IR-120 (H form), and then condensed under reduced pressure below 30°C. Borate was removed as its volatile methyl ester by the addition of methanol. This



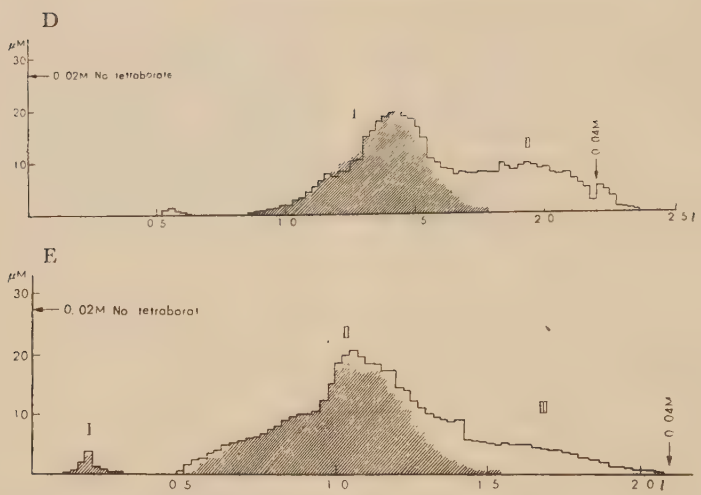


FIG. 4. Chromatography of Pentose on Dowex-1 Borate Column.

Xylose isomerase, A. *L. fermentum*, B. *Leuc. mesenteroides*, C. *L. xylosus*
Arabinose isomerase D. *L. gayonii*, E. *Pc. lindneri*.

▨ Cysteine-carbazole reaction

TABLE VIII PAPERCHROMATOGRAPHY OF FRACTIONS

Organism	Substrate	Fraction No.	R_F ButOH : AcOH : H_2O (4 : 1 : 2)	R_F Phenol : H_2O (9 : 1)	$D_{540} : D_{670}$ in Bial's reaction
L. fermentum	xylose	I	0.39	0.59	0.37–0.40
		II	0.35	0.45	0.19
Leuc. mesenteroides	〃	I	0.40	0.54	0.41
		II	0.36	0.43	0.20
L. xylosus	〃	I	0.40	0.58	0.42–0.43
		II	0.35	0.44	0.20
L. gayonii	arabinose	I	0.45	0.63	0.80
		II	0.35–0.36	0.48	0.19
Pc. lindneri	〃	I	0.40	0.62	0.57
		II	0.42	0.63	0.79
		III	0.34	0.45	0.20

procedure was repeated three times or more, and qualitative examinations were carried out with each fraction by paperchromatography. These elution patterns are presented in Fig. 4, and R_F values of each fraction are listed in Table VIII.

On the xylose isomerization by *L. fermentum*, *Leuc. mesenteroides* and *L. xylosus*, two peaks were completely separated. The first peak revealed a positive cysteine-carbazole reaction, and was identified as xylulose by R_F values of TCA-orcinol sprary (e.g., 0.39 by buthanol : acetic acid : water) and by the ratio of $D_{540} : D_{670}$ in orcinol reaction. Further elution of the column with 0.02 and 0.04 M of borate gave a second peak, and on account that its R_F value was found to be identical with xylose on paperchromatograms, it was identified as xylose by orcinol spectrum although its cysteine-carbazole test was a failure.

Similarly, ribulose was separated by the

first peak and identified by positive cysteine-carbazole test and by orcinol spectrum identical to the authentic sample. The next fraction was ascertained as arabinose. Thus, it was concluded that xylulose is formed from xylose and ribulose from arabinose by these bacteria.

DISCUSSION

At the first stage of fermentation of pentose (D-xylose or L-arabinose) by *L. pentosus* it was suggested that isomerization of pentose to D-xylulose or L-ribulose and formation of their phosphorylated products take place[7,19,24]. Recently, it was confirmed that the fermentation of pentose by this organism involves a novel cleavage of D-xylulose-5-phosphate into acetyl phosphate and glyceraldehyde-3-phosphate[25], and Horecker et al.[26] has established the pathway of pentose fermentation by *L. pentosus*.

Besides this pathway, Fukui et al.[27] have recently demonstrated a new pathway of pentose fermentation by *S. faecalis*, *S. glycerinaceus* and *L. thermophilus* T_1, in which sedoheptulose would presumably be involved. These bacteria and also *L. pentosus* all belong to the homotype. This finding of Fukui et al. is of much interest, because they assumed the occurrence of sedoheptulose as an intermediate of the fermentation product by these organisms, in contrast with Bernstein et al.[28] who have observed that the cross contamination of radioactive carbon is not occurred in the fermentation of l-C^{14}-glucose by *Leuc. mesenteroides*. Activity of transketolase was not detected in the enzymatic preparation of *L. pentosus*.[19] Futhermore, in the phosphoketolase reaction of *L. pentosus*, no acetyl phosphate was formed from such substrates as sedoheptulose-7-phosphate or fructose-6-phosphate.[25] These results revealed that transketolase- and transaldolase-activities are never demonstrated by *L. pentosus* and by *Leuc. mesenteroides*, but these facts are not in consistent with the data of Fukui.

In both cases of *L. pentosus* and of *streptococci* which reveal a new scheme of pentose fermentation, isomerization of pentose may be supposed to take place in the first step of fermentation of xylose or arabinose.

In the present paper, activities of petose isomerases were demonstrated by the heterofermenters. No Sedoheptulose was formed by all of these bacteria from pentose, even when prolonged incubation was instituted. The enzymatic activities were in good accordance with the ability of fermentation of pentose. Some typical examples are presented as follows: *L. fermentum* is able to ferment xylose but not arabinose, and dried cells and cell-free extracts of this bacterium reveal the isomerization of xylose to xylulose, but however do not act on arabinose. With the homofermenters: *L. casei*, *L. acidophilus*, *L. bulgaricus* and *L. delbrückii*, these occurred no acid production from arabinose or xylose, and no evidence for ketopentose formation was obtained. As *L. xylosus* ferments xylose but does not ferment arabinose, hence xylose isomerase is found to be active while not any activity of arabinose isomerase is pointed out with this bacterium. Moreover, among the heterofermenters, two types of xylose isomerase probably exist on the mode of action:

1) Xylulose is formed immediately by ... *L. fermentum*, *Leuc. mesenteroides* > *L. fermenti*,

2) Although the rate of xylulose formation is slow, the amount of xylulose produced is much greater than 1) after prolonged (e.g., 24 hours) incubation of ... *L. brevis*, *L. gayonii* > *L. buchneri*.

But as for arabinose isomerase, its activity is found to be very powerful from four strains of the heterofermenters, and even by an arabinose-fermentable homofermenter: *Pc. lind-*

24) J. O. Lampen, *J. Biol. Chem.*, **204**, 999 (1953).

25) E. C. Heath, J. Hurwitz and B. L. Horecker, *J. Am. Chem. Soc.*, **78**, 5449 (1956).

26) B. L. Horecker, E. C. Heath, J. Hurwitz and A. Ginsberg, *Federation Proc.*, **16**, 198 (1957).

27) S. Fukui, A. Ôi, A. Ôbayashi and K. Kitahara, *J. Gen. Appl. Microbiol.*, (Japan), **3**, 258 (1957).

28) I. A. Bernstein, K. Lentz, M. Mahm. P. Schmbye and H. G. Wood, *J. Biol. Chem.*, **215**, 137 (1955).

neri. Small quantities of ketopentoses are detected from arabinose by such homofermenters as *L. arabinosus* and *L. plantarum*. No ribulose is formed by *L. fermentum* or by *L. fermenti*, since these bacteria do not ferment arabinose.

The pathway of pentose fermentation by heterofermenters may be attributed to Horecker's enzyme system which involves ketopentose and its phosphate compound. So, it may be noted that pentose-isomerization would occur in the first step of xylose or arabinose metabolism by these bacteria.

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A Chromatographical Study of Crystalline Catalase from Bovine Liver

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Since the capability of various catalase activities has been shown with many crystalline preparations, the suggestion that catalase is heterogeneous or is denatured during preparation has been presented by many workers. Using tricalcium phosphate gel prepared by Tiselius, a column chromatography was carried out with crystalline catalase prepared by Shirakawa's method.

After increasing ionic concentration of phosphate buffer by a stepwise elution method, two fractions differed in catalase activity, iron content, and the sedimentation constant eluted from the column. Recovery of the protein concentration was 95% and 5% in accordance with the main and the minor fractions.

INTRODUCTION

Since catalase from bovine liver has been shown to vary in activity according to the preparation[1~3], Sumner et al. studied this point from the view of heterogeneity by using a diffusion test and Twett's column with tricalcium phosphate[4]. The results indicated the homogeneity of crystalline catalase and they suggested that catalase might exist similarly as several different compounds, with regard to protein in one species, but differing in hematin moieties.

Recently, chromatographical studies of proteins have made much progress with many ionic exchanging materials, thus providing a

1) J. B. Summer and A. L. Dounce, *Science*, **85**, 366 (1937).
2) J. B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **121**, 417 (1937).
3) J. B. Sumner and A. L. Dounce, *J. Biol. Chem.*, **127**, 439 (1939).
4) J. B. Sumner, A. L. Dounce and V. L. Frampton, *J. Biol. Chem.*, **136**, 343 (1940).

useful technique in the field of protein chemistry. Tiselius[5] extensively investigated the chromatography of proteins with tricalcium phosphate which has long been known as an adsorbent for protein preparation. Calcium phosphate gel prepared by the method of Keilin et al.[6] which was used for the preparation of catalase was unstable and found to possess many disadvantages, such as flow rate and its reproducibility as an adsorbent of column chromatography. The adsorbent prepared by Tiselius is, however, overwhelmed with such discountered properties, and successful results have been obtained with various proteins, especially high molecular-weight proteins.

By using the calcium phosphate gel of Tiselius, crystalline bovine liver catalase prepared by Shirakawa's method[7] was investigated from the view of its heterogeneity. A twice recrystallized catalase was chromatographed and two components, a main and a minor fractions both having catalase activity, were separated out. Their properties were investigated and will be reported in the following paragraphs.

EXPERIMENTAL

1. Preparation of calcium phosphate gel. Hydroxylapatite, $Ca_5(PO_4)_3OH$, was prepared according to the method of Tiselius. Aqueous solutions of 0.5 M $CaCl_2$ and Na_2HPO_4 were reacted under well stirring. The brushite precipitate was converted into the hydroxylapatite form by the addition of sodium hydroxide and heating. The stabilization of the material was further performed by replication of the heating procedure with phosphate buffer repeatedly. The properties of this material were dependent on pH and temperature during preparation.

2. Assay for catalase activity. Catalase activity was measured according to the method of Euler and Josephson[8]. A diluted enzyme solution of 1.0 ml was pipetted into 50 ml of 0.01 N H_2O_2 in 0.07 M phosphate buffer of pH 6.8 previously cooled at 0°. At the second and 4 th minute, each 5 ml of the solution was pipetted from the reaction mixture and the hydrogen peroxide that remained was titrated with 0.005 N potassium permanganate.

3. Bovine liver catalase. Crystalline bovine liver catalase was prepared by the method of Shirakawa[7]. Fresh bovine liver was water-extracted and fractionated by adding 0.8 vol. of acetone in the cold. The water soluble-fraction obtained from acetone precipitates was separated and fractionated with 0.45 saturation of ammonium sulfate. From the solution fractionated with ammonium sulfate, crude catalase was crystallized out by standing in the cold over night. The crystalline catalase was then recrystallized twice. The Kat. f. value was about 28,000 after assaying by Euler and Josephson's method. Sedimentation studies were carried out with a Spinco ultracentrifuge Medel E which showed ultracentrifugal homogeneity of the preparation: s_{20w} of 10.9 was obtained, this value being in good agreement with the sedimentation reported for other catalases. The preparation was, however, very slightly heterogeneous with Tiselius electrophoresis with M/20 phosphate buffer of pH 7.8 (μ=0.144). The protein concentration was about 0.6 %. (Fig. 1)

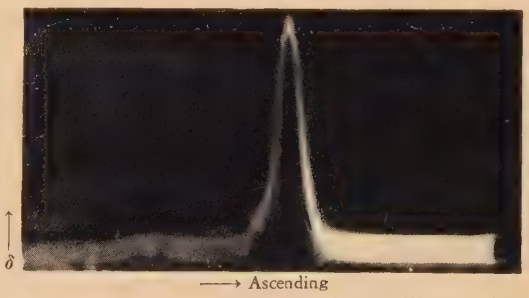

FIG. 1. Electrophoresis pattern of crystalline catalase. Protein concentration: 0.6 %. In phosphate buffer of pH 7.8 (μ=0.144). After one hour.

4. Preparation of column. Small portions of suspension of calcium phosphate were introduced repeatedly into a single glass tube having a diameter of 1.8 cm and a length of 15 cm and were settled under the force of gravity, then with hydrostatic power in order to pack compactly. Glass wool was used to support the adsorbent.

RESULTS

1. Chromatography on calcium phosphate gel. Conditions of chromatography were roughly determined following a preliminary test tube

5) A. Tiselius, S. Hjerten and Ö. Levin, *Arch. of Biochem. & Biophys.*, **65**, 132 (1956).
6) D. Keilin and E. F. Harttree, *Proc. Roy. Soc. Lond. B*, **124**, 397 (1938).
7) M. Shirakawa, *J. Agr. Chem. Soc. Japan*, **23**, 361 (1950).
8) H. von Eluler and K. Josephson, *Ann.*, **452**, 158 (1927).

experiment, which was essentially the same method as adapted by Stein and Moore[9] in their ribonuclease test with IRC 50. Since Tiselius[10] in his study of calcium phosphate chromatography has pointed out that various proteins are eluted out from the column by increasing ionic concentration of phosphate buffer, the experiment was carried out by stepwise elution system, increasing the ionic concentration of phosphate buffer from 0.05 to 0.2 M. Pipetted into the column was 4 ml of about 1% of catalase solution which had been dialyzed against 0.05 M phosphate buffer of pH 6.8. The protein adsorbed on the column was then eluted with phosphate buffers of pH 6.8. Each eluent fraction of 2.5 ml was collected by the use of a fraction collector. Protein concentration was measured by reading optical density at 275 mμ using a Beckman spectrophotometer DU. The Lowry-Folin method[11] was also performed for protein determination and the results were in good accordance with the optical reading at 275 mμ. The following experiments were thus carried out by measuring optical density at 275 mμ.

Fractions were eluted from the column with 0.1 M and 0.2 M phosphate buffers of pH 6.8 respectively. (Fig. 2) The recovery of protein concentration was about 95% and 5% according to the eluting buffers of 0.1 M and 0.2 M. Activity of each fraction was determined, and it was found that enzyme activity was not lost during chromatography.

The Kat. f. values of main and minor components were 30,000 and 10,000 respectively, activity differing in an appreciable rate.

2. Rechromatography of each fraction separated by the column. Each fraction was collected and dialyzed against a phosphate buffer of 0.05 M over night. Condensation was carried out by filtrating the solvent under a weak reduced pressure, the solution being held in a collodion membrane placed in a filtrating flask. Each of the fractions was thus chromatographed in the same conditions as those of original. Since the minor fraction which eluted with 0.2 M phosphate buffer was small in quantity, a large scale of chromatography was required in order to obtain enough amount of this fraction. The two components behaved independently, indicating that no chromatographical denaturation occurred. (Fig. 3)

3. Iron determination. Each of the fractions was dialized against distilled water for one

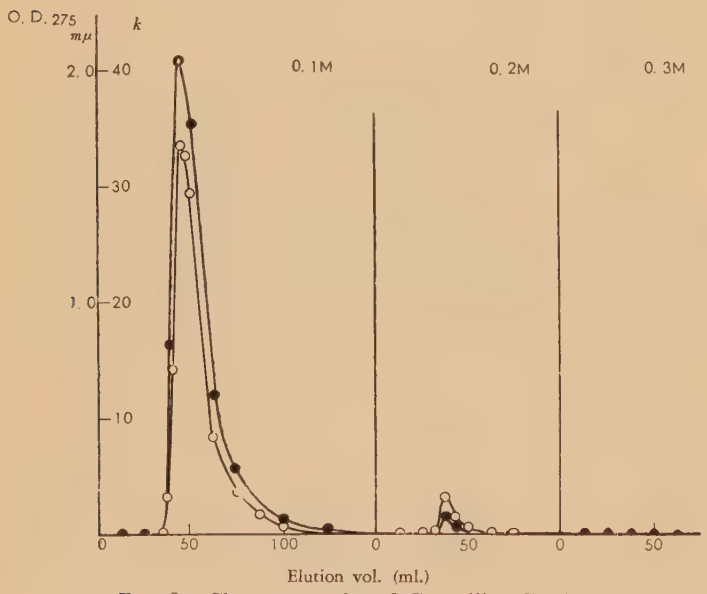

FIG. 2. Chromatography of Crystalline Catalase.
Protein concentration; 45 mg in 4.0 ml of 0.05 M phosphate buffer of pH 6.8. Column; 1.8×15 cm.
○——○: Optical density reading at 275 mμ.
●——●: Catalase activity as the velosity const. of k.
Elution was with 0.1, 0.2 and 0.3 M of phosphate buffers of pH 6.8.

9) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).
10) A. Tiselius, *Arkiv. Kemi*, **7**, 443 (1954).
11) O. H. Lowry, N. J. Rosebrought, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1954).

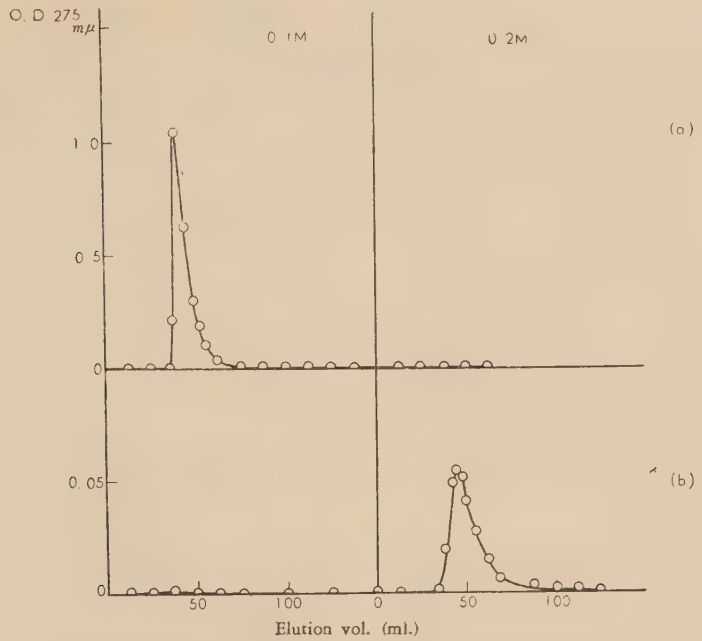

FIG. 3. Rechromatography of Two Fractions.

(a) Chromatography of fraction 1 which came down with 0.1M phosphate buffer in original chromatography. Protein concentration; 14.7 mg in 4 ml of phosphate buffer.

(b) Chromatography of fraction 2 which came down with 0.2M phosphate buffer in original chromatography and concentrated. Protein concentration; 2.5 mg in 10 ml of phosphate buffer.

Remainders; same as in original.

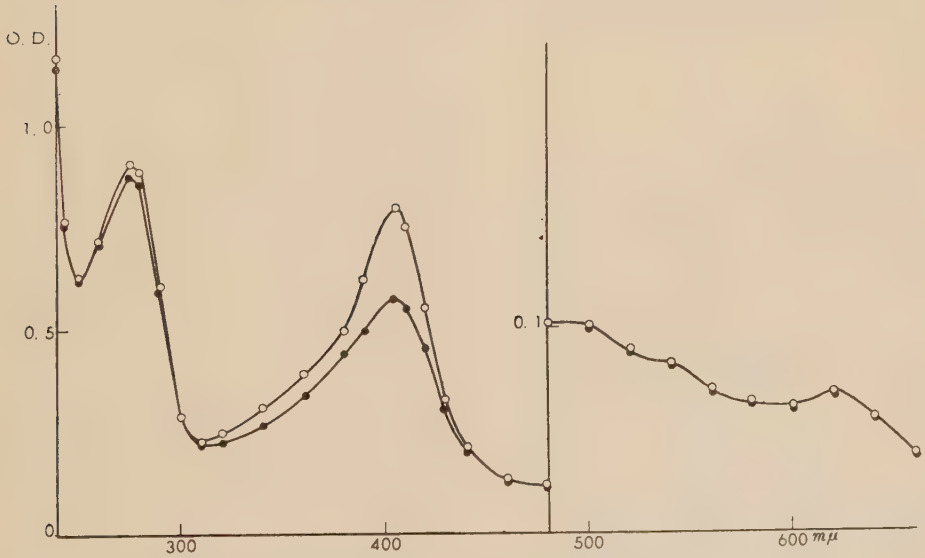

FIG. 4. Absorption Spectrum of Two Fractions Separated by the Chromatography.

○—○: Fraction 1. ●—●: Fraction 2.

week in the cold. The solution were dried and the proteins were decomposed by a huming reagent of H_2SO_4 and H_2O_2. The iron contents were determined by the orthophenanthroline method[12] and by α,α'-dipyridil[13]. The main fraction was shown to contain iron in the amount of 0.11%, whereas iron contained in the minor fraction was 0.08%.

4. Ultracentrifuge. The two fractions were investigated by ultracentrifuge, using a Spinco Model E and sedimentation constants were calculated from the Svedverg equation. The partial specific volume of 0.74 given by Deutsch[14] was used. The main component showed its s_{20w} as 11.0, and that of the minor component was 8.9. A single peak was shown in each component.

5. Spectrophotometric data. The optical absorption spectrum of the fractions was measured by means of a Beckman spectrophotometer. (Fig. 4) Hematin moiety of the fractions was quite agreeable, whereas the absorption at 405 mμ which was due to the binding of protein and heme was characteristically different. The minor fraction showed remarkable reduced absorption in this range. The ratio of D_{405}/D_{280} was thus 0.91 and 0.60, corresponding to both main and minor components.

DISCUSSION

Bovine liver catalase prepared by Sumner varied in its Kat. f. from 28,000 to 42,000, and catalase prepared by Shirakawa's method showed Kat. f. in the range of 30,000 to 32,000. Various activities of erythrocyte were also reported[15~17]. Deutsch[18] suggested the denaturation of catalase during the preparation for such capability of crystalline catalase activity, and his kinetic studies indicated the heterogeneity of catalase. An attempt by the present author to investigate crystalline catalase using a column chromatography was made and the catalase was separated into two fractions.

The problem whether these two fractions are induced artificially is not certain. However, there is little doubt that each fraction is chromatographically homogeneous and these fractions are not produced during chromatography, since these two fractions behaved independently on rechromatography.

Stein[19] observed that during the lyophilic storage of ribonuclease at 0°C for 16 months the enzyme was transformed into a minor component which was chromatographically distinct from the original and enzymatically active. It was also reported that the transformation of lysozyme carbonate leads to a new enzymatically active component when it is allowed to stand at room temperature in a lyophilized state. The catalase preparation used in this experiment was stored at 0° to 5° as a concentrated solution for a period over several weeks. After treatment with chromatography, the proportion of the main and the minor components, however, did not change in an appreciable rate. The presence of the minor components is thus supposed not to be derived during storage period.

Greenfield and Price[20] demonstrated four fractions from s ˙ıble catalase fraction of normal and tumour bearing rat liver, using calcium phosphate-cellulose gel column. Since chromatography was carried out with several preparations of bovine liver catalase in this experiment and reproduceable chromatography was obtained, the presence of more than one catalase species is thus predicted in normal bovine liver similar to rat liver, assuming that no denaturation occurs during preparation. By repeating recrystallization, the minor fraction should be separated, since electrophoresis showed a significant difference between the two components.

12) G. Barkan and S. Walker, *J. Biol. Chem.*, **135**, 37 (1940).
13) Lingel, *Z. Ges. Exp. Med.*, **86**, 269 (1933).
14) H. F. Deutsch, *Acta Chemica Scand.*, **6**, 1516 (1952).
15) R. K. Bonnichsen, *Arch. Biochem.*, **12**, 83 (1947).
16) D. Herbert and J. Pinsent, *Biochem. J.*, **43**, 203 (1948).
17) H. F. Deutsch, *Acta Chem. Scand.*, **5**, 815 (1951).
18) H. F. Deutsch, *Acta Chem. Scand.*, **5**, 1074 (1951).
19) W. H. Stein, *Adv. in Prot. Chem.*, **XI**, 210 (1956).
20) V. E. Price and R. E. Greenfield, *J. Biol. Chem.*, **209**, 363 (1954).

Optical absorption of these components indicated the different behavior of binding of heme and protein. Hematin moiety was, however, incident in two components. The lower catalase activity of the minor component may be considered to be due to protein specificity and also the lower ratio of D_{405}/D_{280} was suggestive of the reason of lower activity.

It would be an interesting problem to study other catalase preparations from the view of heterogeneity, by means of a chromatographical technique.

SUMMARY

(1) Crystalline catalase prepared by Sirakawa's method was separated into two components, by application of the column chromatography with tricalcium phosphate gel of Tiselius. By stepwise elution, changing ionic concentration of phosphate buffer of pH 6.8, a main and a minor components eluted from the column with 0.1 and 0.2 M.

Protein recovery was 95% and 5%, and Kat. f. was 30,000 and 10,000, respectively, in accordance with main and minor components.
(2) These two components behaved independently when rechromatography was performed, indicating that no denaturation occurred during chromatography.
(3) Sedimentation constant was $s_{20w}=11.0$ and 8.5, and the iron content was 0.11 and 0.08%, respectively, in accordance with the main and the minor fractions.
(4) The optical absorption at hematin moiety showed no significant difference between the two components, whereas at 405 mμ the minor component characteristically decreased in its absorbancy.

Acknowledgement. The author wishes to express sincere thanks to Prof. Y. Oshima for his encouragement and interest shown in this work and to Mr. Hayashi for his assistance in ultracentrifuge work.

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On the Formation of Higher Alcohols in the Fermentation by Yeasts

The Formation of Amyl Alcohol in the Mediums which contain Sugar and Nitrogen Compounds except Leucine

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When sugar mixed with certain nitrogenous compounds other than leucine is fermented by yeasts, a small quantity of amyl alcohol is always obtained. We have examined this mechanism and concluded that amyl alcohol is produced from leucine which is caused from the decomposition of yeast protein. The decomposition products of yeast protein also contain valine, but no trace of iso-butyl alcohol was detected after fermentation.

It was one of the most important findings at the time when synthetic saké came out its first appearance, that when sugar solution with alanine was fermented by yeasts, a flavour similar to that of saké was produced. This was afterwards proved to be due to the formation of iso-butyl alcohol by one of the authors M. Yamada[1]. Though isobutyl alcohol should result from valine according to F. Ehrlich's scheme, the fermentation of valine was not so easy and no trace of iso-butyl alcohol could be detected by M. Yamada[2] even when the fermentation of valine did occur. Later on Y. Tomoda[3] and R. Thorne[4] reported some facts opposite to this.

Apart from these findings, when sugar mixed with some nitrogenous compounds other than leucine was fermented by yeasts, a small quantity of amyl alcohol was always obtained, but this mechanism has not yet been made clear. In spite of this, it is supposed that the alcohol may result from amino acid produced by the decomposition of yeast protein which was previously reported to contain valine, leucine and glutamic acid etc.

Thus, we may possibly explain the mechanism of higher alcohol formation through studying the fermentation of sugar mixed with yeast decomposition products by yeasts.

On the fractional distillation of the oily substance obtained we could not gain a fraction distilling at about 108°C, but only a fraction distilling over 120°C, which was proved to contain amyl alcohol. From these facts we can conclude that amyl alcohol was produced from leucine which was not added to the medium but resulted from the decomposition products of yeast protein. The decomposition products of yeast protein also contain valine, but no trace of iso-butyl alcohol could be detected after fermentation. Thus, valine did not seem to be a direct source of iso-butyl alcohol. At the same time, a considerably large amount of succinic acid was produced by this fermentation.

EXPERIMENTAL

1. The fermentation in the medium containing sugar and hot water extract of yeasts.

(1) Preparation of yeast extract: Two kg. of baker's yeast manufactured by Oriental Yeast Co., Ltd. was put in a round-bottomed flask; 10 l of water was added and the mixture was heated for three hours

1) M. Yamada, This Bulletin, **8**, 95~100, (1932).
2) M. Yamada, This Bulletin, **11**, 24~26, (1935).
3) Y. Tomoda, *J. Chem. Ind. Japan*, **42**, 806~809, (1939).
4) R. Thorne, *J. Inst. Brewing*, **43**, 288, (1937).

in a boiling water bath. The filtrate obtained amounted to 64 l.

From the results of applying paper chromatography to the filtrate, fairly large amounts of valine and leucine were detected ih the filtrate.

(2) Fermentation and distillation: The solution containing 6.4 kg of sugar, 64 l of the filtrate, $MgSO_4$ and KH_2PO_4 was pasteurized and fermented by the same yeasts for 10 days at 25°C.

The fermented solution was filtrated and then distilled. After repeated distillation the concentrated alcoholic solution was fractionated with Widmar's column. Table I shows the fractionated alcoholic solution.

TABLE I

fraction	concentration of alcohol (%)	volume (ml)	vanilline-H_2SO_4 reaction
1.	94	160	blue
2.	94.5	210	yellow
3.	94	2860	yellow
4. fusel oil		3.6 g	reddish purple

(3) Fractional distillation of fusel oil: Fusel oil fraction was further divided, as shown in Table II.

TABLE II

fraction distilling at °C	yield (g)	m. pt. of 3,5-dinitro-benzoate (DNB) (°C)
91~100	0.1	
100~112	0.1	
112~118	0.1	48
118~125	0.4	48
125~128	0.8	
128~130	1.5	60
residue	0.6	

Each DNB was subjected to the method of silica-gel column chromatography reported by White and Dryden[5]. Table III shows the results.

TABLE III

fraction distilling at °C	number of band	position of band	m.pt. of extract (°C)
112~118	2	low	51.5
		high	not determined
118~125	2	low	53
		high	75
125~130	1		59

Table IV shows the results of analysis of fraction distilling at 128~130°C.

TABLE IV

Found: N, 9.49%. Calcd. for $C_{12}H_{14}O_6N_2$: N, 9.93%

The fraction of 128~130°C was determined as iso-amyl alcohol and the two other fractions were conceived to be a mixture of iso-amyl alcohol and ethyl alcohol.

(4) Acids: From the residue of distillation we obtained 21 g of succinic acid and 5.5 g of zinc lactate which contained 3 molecules of crystallized water.

II. Fermentation in the medium which contains sugar and acid hydrolysate of yeasts.

(1) Preparation of hydrolysate of yeasts with hydrochloirc acid: A mixture of 5 kg of baker's yeast manufactured by Oriental Yeast Co., Ltd. and 5 l of conc. hydrochloric acid was heated in a round-bottomed flask in the water bath, until a large amount was dissolved and it was then boiled with a reflux condenser for about eight hours. The products of hydrolysis were filltered and the filtrate was filled up to 1500 ml The nitrogen content of the solution was 69.346 g/l.

From the results of applying paper chromatography to the solution, fairly large amounts of valine and leucine etc. were detected.

(2) Composition of the medium: Table V indicates the composition of the medium.

TABLE V

Sucrose	100 g
Solution of yeast hydrolysate	8 ml
$MgSO_4 \cdot 7H_2O$	3 g
KH_2PO_4	1 g
Distilled water	1 l
pH	5.6

The total volume was 50 l. The yeast used for fermentation was distillery yeast-Hakken No. 1.

(3) Fermentation and distillation: Two ml of yeast suspension precultured for five days, was added to each 1 l of the medium; the mixture was fermented for ten days and filtered and then the filtrate was distilled.

(4) Determination of alcohols: The distillate was fractionated and divided into three parts as shown in Table VI.

TABLE VI

fraction	alcohol (%)	volume (ml)	vanilline-H_2SO_4 reaction
1.	95.1	560	blue
2.	95.7	1700	yellow
3. fusel oil (b.pt. over 82°C)		26.5g	reddish purple

5) J. White & E. Dryden, *Anal. Chem.*, **20**, 853, (1948).

Fraction 3 was further distilled and divided as shown in Table VII.

TABLE VII

fraction distilling at °C	volume (ml)	m.pt. of 3,5-dinitrobenzoate (DNB) (°C)
100~112	trace	
112~118	0.12	50
118~125	0.76	55
125~131	2.35	60~61
residue	0.05	

We applied the method of silica-gel column chromatography (White and Dryden) for each DNB derivative. The results are as shown in Table VIII.

Analysis of DNB of the fraction distilling at 128~130°C. Found: N, 9.59%. Calcd. for $C_{12}H_{14}O_6N_2$: N, 9.93%.

The alcohol fraction of 128~130°C was identified as iso-amyl alcohol and the two other fractions were considered as a mixture of ethyl alcohol and iso-amyl alcohol.

TABLE VIII

fraction distilling at °C	number of band	position of band	m.pt. of extract (°C)
112~118	2	low	55
		high	79
118~125	3	low	53
		middle	49
		high	not determined
128~130	1		59

(4) Acids: From the residue of distillation we obtained 30.6 g of succinic acid (m.pt. 184°C) and 20.6 g of zinc lactate which contained 3 molecules of crystallized water.

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Studies on Mitochondriogenesis during Plant Germinating Period

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Mitochondrail particles of embryos and endosperms increase in their amounts and number at an earlier germinating period. The newly-born mitochondrial particles which were weak in their activity, gradually come to possess increased activity. In cytoplasm, it is observed that dehydrogenase activities linked to DPN and TPN increase prior to the establishment of mitochondria.

Many studies have been made on Mitochondria since Hogeboom first isolated this substance from animal origins, but yet very few reports have appeared on the process of the generation of mitochondria—mitochondriogenesis. Attempting to investigate the mitochondriogenesis biochemically, we studied on the increase of mitochondrial particles and their enzymatic activities during the germination of seeds, in relation with dehydrogenase activities of the cytoplasmic proteins.

Some results obtained from etiolated seedlings of wheat and radish are reported in this paper, and mitochondriogenesis has been

discussed.

EXPERIMENTAL

(1) Glucose-6-phosphate and Pyridine Nucleotides. The barium salt of glucose-6-phosphate was prepared according to the method of Inoue et al.[1], and was convertes into sodium salt before use. DPN was isolated from baker's yeast by the method of Le Page[2], and TPN from pig liver according to Okunuki's methods[3].

(2) Seedlings. The etiolated seedlings of wheat (alubuminous seed) and radish (exalbuminous seed) were prepared as follows: In advance of seedling, the seeds selected out according to their size, were treated with 70% alcohol for 2 minutes and further soaked in 3% H_2O_2 for 2 minutes, and washed thoroughly with distilled water. The sterilized and the washed seeds were allowed to germinate for about 3 days in the dark on wet filter paper at 30°C, till the radicles attained a length of 20 mm.

The embryos and endosperms of wheat seedlings were separated by cutting with a scalpel. Each of the tissues was thoroughly homogenized using a homonizer of 0.1 N phosphate buffer 3 times in volume, pH 7.5, including 15 per cent sucrose and 3×10^{-2} M/EDTA. The entire procedure was carried out at 5°C.

The homogenates thus obtained, were filtered through a cheese cloth and centrifuged at 4,800 g for 10 minutes. The sediments were discarded and the supernatants, which were designated as "Juice," were centrifuged for 20 minutes at 10,800 g. The sediments were suspended in an appropriate volume of phosphate buffer, and centrifuged again.

The sediments were designated as the "Mitochondrial Fraction." The designation "Soluble Fraction" was given to the supernatants from "Juice".

(3) Turbidometrical and Phase and Construct Microscopic Studies on Mitochondrial Fractions. Mitochondrial Fractions were suspended in an appropriate volume of the buffer solution, and the turbidities of the suspensions were measured using a spectrophotometer (430 mμ). The number of the mitochondrial particles of the suspensions in the visual field were counted under a phase contrast microscope of 800 magnifications, and is expressed as "number" in Tables I and II.

(4) Determination of Dehydrogenase Activity in Soluble Fraction. The Soluble Fractions were dialized to remove the substrates, and the TPN-linked dehydrogenase activities were estimated as follows: 2.0 ml of the dialized enzyme, 0.2 ml. 0.1 M G-6-P, 0.3 ml 2×10^{-2} M TTC, 2.2 ml. 1.5×10^{-2} M $MgCl_2$, 0.1 ml 0.01 per cent methylene blue, 0.2 ml TPN solution (5 mg/ml water) or 0.2 ml DPN (0.6 mg/ml.) plus 0.2 ml. 0.01 M ATP in place of TPN, and the phosphate buffer were mixed up to 3.0 ml, and the reaction was permitted anaerobically for 60 min. at 30°C. The reaction was stopped by the addition of trichloracetic acid, and the formazone formed was extracted in 5 ml of ethyl acetate, and determined spectrophotometrically (480 mμ).

(5) Determination of Dehydrogenase Activity and Oxygen Uptake in Mitochondrial Fraction. The O_2-uptake of Mitochondrial Fraction was measured manometrically with succinate and malate as substrates under the condition mentioned in the following Results and Discussion.

RESULTS AND DISCUSSION

(1) Turbidities and Numbers of Particles of Mitochondrial Fractions. The turbidities of Mit. Fracts. separated from wheat and radish seedlings increased in proportion to germinating-age. The number of particles in the fractions also showed the same tendency as that of the turbidity.

TABLE I

TURBIDITY AND NUMBER OF MITOCHONDRIA SEPARATED FROM EMBRYOS AND ENDOSPERMS OF WHEAT SEEDLINGS

Seedling Ages (hours)	Embryos		Endosperms	
	Turbidity	Number	Turbidity	Number
24	0.256	40~ 50	0.215	13~20
48	0.417	60~ 65	0.352	43~58
72	0.469	76~116	0.432	—
96	0.606	120~	0.444	—

Mitochondria of Embryos separated from 8 gm of the dry seeds, were suspended in 10 ml of Sucrose-Phosphate Buffer. Mithochondria of Endosperms separated from 2 gm of the dry seeds, were suspended in 24 ml of the same buffer.

From the above relationships the turbidities were considered to be representative of the number, in the following experiments. From Tables I and II, it is suggested that the mitochondrial particles increase in number

1) Inoue et al., *J. Agr. Chem. Soc. Japan* (in Japanese), **30**, 59 (1956).
2) Le Page, G. A., *J. B. C.*, **176**, 1021 (1948).
3) Okunuki, *Methods in Enz.* (in Japanese), **2**, 682 (1955).

during 48~72 hours.

TABLE II
TURBIDITY AND NUMBER OF MITOCHONDRIA SEPARATED FROM WHOLE RADISH SEEDLINGS

Seedlings Ages (hours)	Turbidity	Number
3	0.201	25~ 42
24	0.201	30~ 37
48	0.222	50~ 60
72	0.310	—
96	0.530	146~164

Mitochondria separated from 2 gm of the dry seeds, were suspended in 24 ml of Sucrose Phosphate Buffer.

(2) Enzyme Activity of Mitochondrial Fraction. In order to investigate whether the particles which increased in their amounts, actually exhibit the enzymatic behavior of mitochondria, the increase of oxygen uptake of the Mitochondrial Fractions was measured manometrically with some of the citric acid cycle acids.

TABLE III
OXYGEN UPTAKE OF MITOCHONDRIA SEPARATED FROM EMBRYOS AND ENDOSPERMS OF WHEAT SEEDLINGS

Seedling Age (hours)	Embryos		Endosperm	
	Malate	Succinate	Malate	Succinate
24	—	0.8	—	3.3
48	11.7	14.7	4.1	6.7
72	20.0	16.1	7.5	8.7
96	28.5	24.8	15.0	14.8

Reaction mixture: 2.0 ml of Mitochondrial suspension, 0.2 ml of 1.5×10^{-2} M $MgCl_2$, and 0.4 ml of 0.05 M of substrate solution. The mitochondria of embryo separated from 8 gm of the dry seeds, were suspended in 10 ml of sucrose phosphate buffer. The mitochondria of endosperms separated from 4 gm of the dry seeds, were suspended in 12 ml of same buffer.

TABLE IV
OXYGEN UPTAKE PER TURBIDITY OF MITOCHONDRIA OF WHEATS SEEDLINGS

Seedling Age (hours)	Embryos		Endosperm	
	Malate	Succinate	Malate	Succinate
24	—	3.1	—	15.3
48	28.0	35.3	11.6	19.0
72	42.6	34.3	17.4	20.1
96	47.0	40.9	33.7	33.3

The ratios of the amounts of oxygen uptake to the turbidities, that is, the oxygen uptake per unit particle, increased with germinating-age, suggesting that the newly-born mitochondrial particles, which were weak activity, gradually come to possess increased enzymatic activity.

(3) Dehydrogenase Activity of the "Soluble Fraction". In order to study any change occurring in the cytoplasm during the increase in the mitochondria mentioned above, TPN and DPN linked dehydrogenase activities of dialyzed Soluble Fractions were estimated.

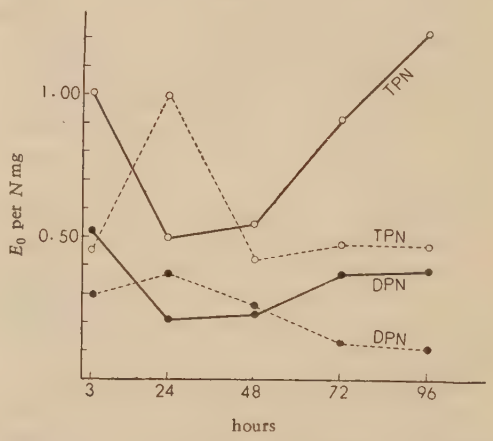

FIG. 1. Activity of TTC Reduction in Soluble Fraction of Embryos and Endosperms separated from Germinating Wheat Seedlings.
Embryo, solid lines; Endosperm, broken lines.

The dehydrogenase activities of the Soluble Fractions from the endosperms attained a maximum after 24 hours, thereafter gradually showing a decrease. In contrast to this, the fractions from 24-hour-old embryos showed a minimal activity, and then increased their activities almost in proportion to age.

It is a well acknowledged fact that the endosperm supplies the embryo with substrates at the first-stage of germination. So, to investigate this and discuss the results shown in Fig. 1, changes of the amounts of glucose in both tissues were investigated.

As shown in Table V, the amounts of sugar in the endosperm maintained a constant level after 72 hour, germination, but those in

TABLE V
CHANGE IN THE AMOUNTS OF WATER SOLUBLE REDUCING SUGAR WITH AGE OF WHEAT SEEDLINGS

Seedling Age (hour)	Embryo	Endosperm
3	1.3	3.3
24	1.6	3.0
48	7.3	2.8
72	28.1	5.1

The results were expressed in mg of glucose per 1 gm of dry seed.

the embryo, which maintained the level of 1~2 mg/g for the first 24 hours, rapidly increased during the latter 2 days.

In the seedlings of radish which is one of the exalbuminous seeds, these relations were found to be more, that is, the dehydrogenase activities markedly increased after 24 hours.

In all cases, the dehydrogenase activity of cytoplasm increased after 24 hours, but the amounts of mitochondria increased after 48 hours. Thus, the cytoplasmic dehydrogenase activity changed prior to mitochondriogenesis.

From Figs. 1 and 2, it is shown that with both seedlings, the TPN linked dehydrogenase activity prefers the DPN linked one. Supposing that the TPN and DPN linked

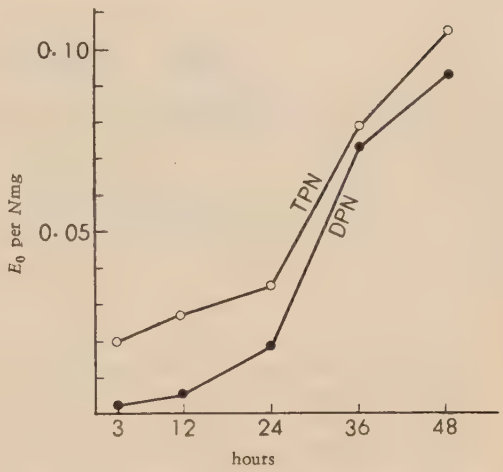

FIG. 2. Activity of TTC Reduction in Soluble Fraction of Germinating Whole Radish Seedlings.

dehydrogenase activities are representable of the activities of oxidative and glycolytic paths respectively, it might be stated that the synthetic reactions are more active than the energy producing reactions in the cytoplasmic proteins of the germinating seedlings. These considerations may present a complete explanation of the increase in the amounts of mitochondria shown above.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 320~324, 1958]

Nature of Lysolecithin in Rice Grains

Part I. Lysolecithin as a Constituent of Non-Glutinous Rice Grains

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Received June 10, 1958

In 1930, Iwata isolated lysolecithin from polished rice grains. Though Iwata proved that the oral administration was not harmful, the biological significance of the presence of lysolecithin in rice grains was a problem left unsolved for a period covering two decades. It was not made clear whether lysolecithin was a constituent of rice grains, or was formed from lecithin naturally or artificially either in storage or during the isolation procedure. Neither was the location of lysolecithin in the grains determined. This article makes it clear that lysolecithin is a normal constituent of non-glutinous rice grains, being formed during the stage of ripening, and is practically unchanged during storage. Lysolecithin is present exclusively, in the endosperm of non-glutinous rice grains, but is not present in glutinous grains.

I. INTRODUCTION

The presence of lysolecithin, a powerful hemolytic agent, in rice grains was demonstrated by Iwata[1] in 1930 who isolated it in a crystalline form and proved that the fatty acid residue is composed of palmitic acid. The same author proved that oral administration was not harmful. Hirao[2] studied the distribution of lysolecithin in plants, and found this substance to be present in non-glutinous seeds of cereals.

While the problem of lysolecithin in plants has been neglected for some decades, the chemistry of lipid and phospholipase has made great advances, and the significance of phospholipid in mitochondria has become one of the most important problems of recent biochemistry.

It is reported in this paper that lysolecithin is one of the normal constituents of the endosperm of non-glutinous rice grains, because we have observed that the amounts of lysolecithin increase in proportion to the ripening of the grains, remain constant in storage, and decrease in germination.

1) M. Iwata, *J. Agr. Chem. Soc. Japan*, **6**, 759 (1930).
2) S. Hirao, *ibid.*, **7**, 364 (1931).

II. MATERIALS AND REAGENTS

a) Rice Grains: Several cultivated species of rice grains were granted from the Food Research Institute in Tokyo, and the Tochigi-ken Agricultural Experiment Station. Prematured rice seeds were obtained from the National Institute of Agricultural Science, Nishigahara, Tokyo, and from the Experimental Farm attached to the University of Tokyo.

b) Standard lecithin and lysolecithin: Standard lecithin was prepared from egg yolk after the method of Pangborn[3]. Standard lysolecithin was prepared by hydrolyzing lecithin with phospholipase A from snake venom after the method of Hanahan et al.[4]

III. METHODS

Powdered rice grains were extracted with ethanol and the ethanol solution was concentrated to a certain volume, the amounts of lecithin and lysolecithin were determined semiquantitatively by means of paper chromatography.

In general, 20 g of either whole or polished rice grains was powdered, and the flour was extracted three

3) M. C. Pangborn, *J. Biol. Chem.*, **188**, 471 (1951).
4) D. J. Hanahan, M. Rodbell, and L. D. Turner, *J. Biol. Chem.*, **206**, 431 (1954).

times with 30 ml. of 90 % ethanol at 50° for 24 hours. The ethanol solution was concentrated in vacuo in an atmosphere of carbon dioxide. About 10 ml. of a mixture of chloroform and ethanol (1 : 1) was added to the syrup and the resulting precipitate was centrifuged off. The solution was concentrated to about 2 ml., to which 10 ml. of acetone was added. The mixture was then left at 0~5° for several hours, and the resulting precipitate was collected by centrifuge. The precipitate was filled up to 1 ml. with ethanol and was used as the sample for chromatography.

One μl. of the sample was spotted on a sheet of filter paper, Tôyô No. 50, impregnated with silicic acid,[5] and developed upward with a solvent of isoamylalcohol : pyridine : acetone : water =8 : 2 : 1 : 1.

Spots of lecithin and lysolecithin were detected by phosphomolybdate and stannous chloride according to the method of Levine et al,[6] and the optical densities at 675 mμ of the spots were measured with a Hitachi spectrophotometer. The R_F values of lecithin and lysolecithin were 0.50 and 0.28, respectively. The total amount of choline was determined by the method of Appleton et al.[7]

IV. EXPERIMENTS AND RESULTS

A. Preliminary experiments. The possibility that lysolecithin might be formed during the process of extraction was denied as a result of the following experiments.

In the first place, phospholipase A was inactive in ethanol solution under the conditions applied for the isolation of lysolecithin.

Secondly, when the extraction process was performed by adding 40 mg of lecithin to 20 g of rice, that means, the concentration of lecithin was increased about ten times as much as that of the natural concentration, the added lecithin was quantitatively recovered, while the amount of lysolecithin was not increased. (Fig. 1)

B. Assay of lecithin and lysolecithin in maturing grains. Amounts of lecithin and lysolecithin in maturing rice grains were assayed. Each twenty grams of fresh rice grains was harvested 10, 20, 30, and 50 days after flowering, and employed as the material. The data in Fig. 2 and Table I indicate that the amount of lysolecithin increases in proportion

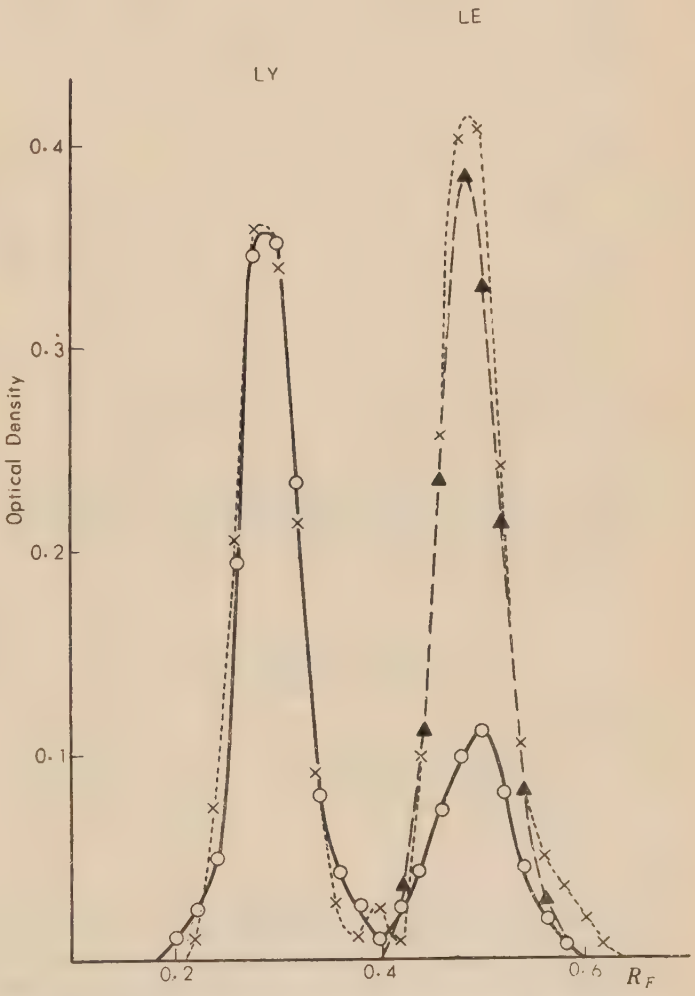

FIG. 1. Paper chromatograms which indicate that lecithin was not hydrolyzed by rice flour in ethanol solution.
LY: Lysolecithin, LE: Lecithin
○—○: Natural rice flour ×-----×: Natural rice flour plus lecithin
▲-----▲: Lecithin.

5) C. H. Lea, D. N. Rhodes and R. D. Stoll, *Biochem. J.*, **60**, 353 (1955).
6) C. Levine, & E. Chargaff, *J. Biol. Chem.*, **192**, 465 (1951).
7) H.D. Appleton, B. N. La Du, B. B. Levy, J. M. Steele, and B. B. Brodie, *J. Biol. Chem.*, **205**, 803 (1953).

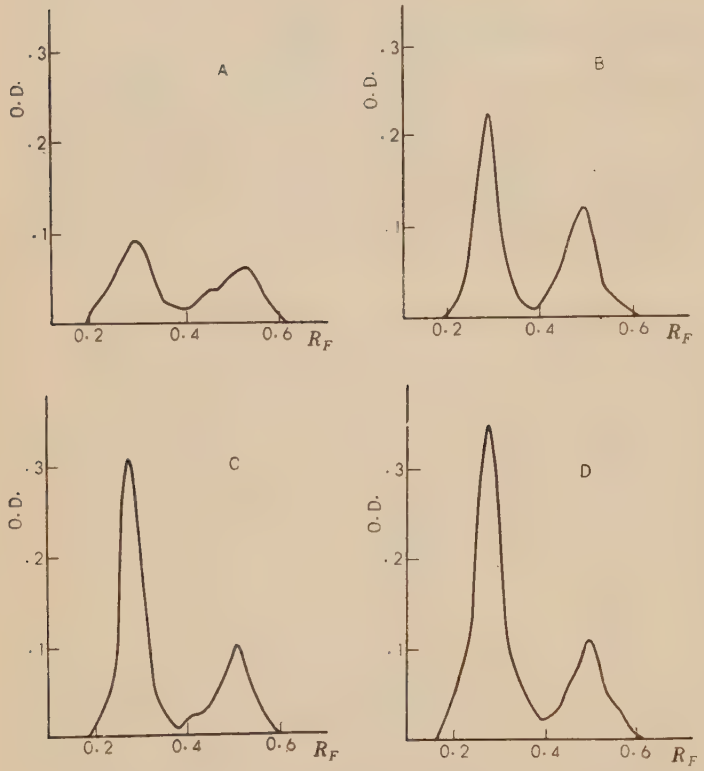

FIG. 2. Paper chromatograms of lysolecithin and lecithin in maturing grains. A, B, C, and D indicate the amounts of lysolecithin and lecithin in the grains 10, 20, 30, and 50 days after flowering.

TABLE I
AMOUNTS OF TOTAL CHOLINE AND STARCH IN RIPENING RICE GRAINS

Days after flowering	10	20	30	50
Total choline (mg/20 g wet-weight)	2.15	5.10	6.25	6.31
Dry weight (g/20 g wet-weight)	5.9	12.7	13.5	17.2
Total choline/dry weight	0.38	0.40	0.46	0.39

c.f. Fig. 2.

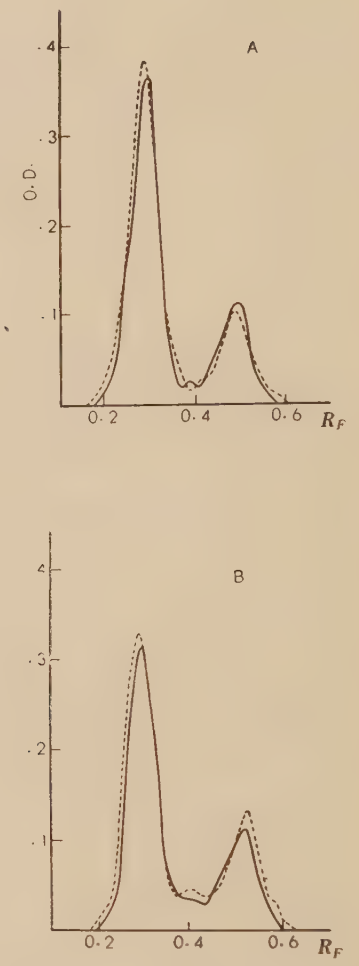

FIG. 3. Paper chromatograms of lysolecithin and lecithin in fresh and stored rice grains.

A: Norin No. 1,
B: Norin No. 29,
———: Fresh one,
·····: Stored one.

to the ripening of the grains.

C. Assay of lysolecithin in fresh and stored rice grains. Amounts of lecithin and lysolecithin in fresh grains, which were harvested about a month in advance, and in stored grains which were stored for about a year were analysed. Their amouts and the relative amounts were not changed significantly during storage of any of the 18 cultivated species. Two examples are given in Fig. 3. Glutinous rice, however, does not contain lysolecithin as it was already been reported by Hirao.[2] (Fig. 4)

D. Assay of lysolecithin in seedlings. Each 20 g of rice-seeds was sowed in each petri-dish, and the whole seedlings in a petri-dish were analysed at intervals. As it is shown in Fig. 5, the amounts of both lecithin and lysolecithin decrease as germination proceeds.

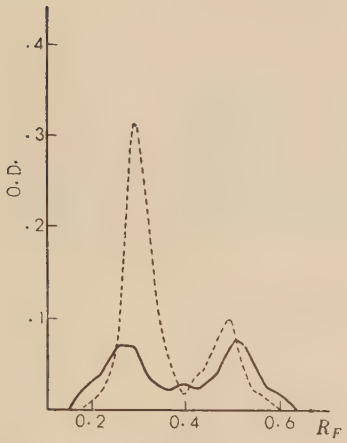

FIG. 4. Paper chromatograms of lysolecithin and lecithin in glutinous and non-glutinous rice grains.
———— : Glutinous rice (Saitama Mochi No. 10),
- - - - - - : Non-glutinous rice (Norin No. 6).

E. Assay of lysolecithin in various parts of grains. Each 20 g of whole rice grains were polished to obtain "polished rice with embryo sperm" and "completely polished rice". The paper chromatograms of the fractions are shown in Fig. 6. Almost as much lecithin and lysolecithin as in the whole grains are present in "completely polished rice grains", indicating that both lecithin and lysolecithin are present almost exclusively in the endosperm of rice grains.

V. DISCSSION AND CONCLUSION

A. Lysolecithin is not an artifact. Although it was reported that phospholipase A and B were present in rice grains[8], the possibility that lysolecithin might be formed during the extraction procedure from lecithin is denied almost certainly from our preliminary experiments.

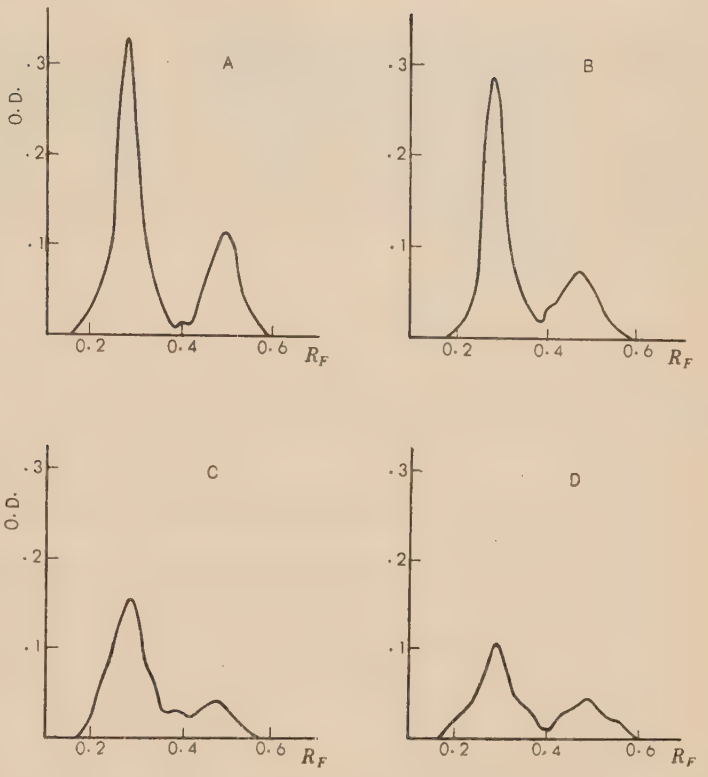

FIG. 5. Paper chromatograms of lysolecithin and lecithin in rice plant seedlings. A, B, C, and D indicate the amounts of lysolecithin and lecithin in 4, 8, 12, and 20 days old seedlings.

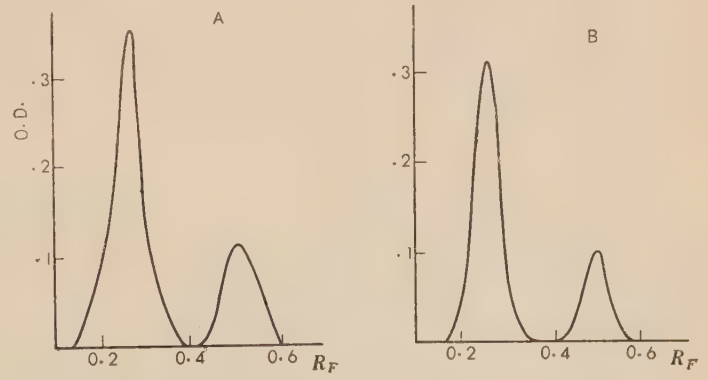

FIG. 6. Paper chromatograms of lysolecithin and lecithin in whole grains (A) and in endosperm of rice grains (B).

B. The amount of lysolecithin does not change in storage. Lysolecithin is formed in the grains at the stage of ripening, and the amount

8) A. Contardi and A. Ercoli, *Biochem. Z.*, **261** 275 (1933).

does not change on storage. But the amount gradually decreases in germination. The metabolic pattern of lysolecithin in germination is a problem open to further investigation.

C. Lysolecithin is a component of the endosperm of rice grains. Both lecithin and lysolecithin are present in the endosperm of non-glutinous rice grains.

VI. Acknowledgement

Our thanks are due to Professor Y. Togari of the University of Tokyo, Dr. T. Tani of the Food Research Institute, Mr. S. Miyake of the Tochigi-ken Agricultural Experiment Station, and Mr. Y. Tsukano of the National Institute of Agricultural Science, Nishigahara, who kindly granted us several kinds of rice grains.

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Nature of Lysolecithin in Rice Grains

Part II. Complex Formation of Lysolecithin with Starch

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Problems of the interaction between starch and lysolecithin are treated in this article. The endosperm of rice grains was fractionated and it was found that lysolecithin was associated with starch granules. The association was not a mere adsorption, but rather a more firm combination. It was demonstrated that lysolecithin had an ability to combine with starch in aqueous solution, and to cause amylose to precipitate, but not amylopectin. The lysolecithin-amylose complex was isolated, and it was shown that lysolecithin is most certainly included in the helicoidal structure of amylose chain. A possible role of lysolecithin during the course of the starch formation in rice grains is discussed.

I. INTRODUCTION

It was reported in the previous report[1] that lysolecithin is contained in the endosperm of non-glutinous rice grains, and it is now shown that lysolecithin is associated with starch granules in the endosperm of the grains.

Although the intrinsic linkage of lysolecithin has yet not been clarified, it was observed that lysolecithin froms a complex with starch in solution, and causes amylose to precipitate, leaving amylopectin in the solution. The lysolecithin can be extracted from the complex with hot ethanol, but at room temperature, only slightly, as in the case of the natural starch granules.

Several substances such as butanol[2], isoamyl alcohol[3], and fatty acids[4,5,6] are known to precipitate amylose from a starch solution,

1) A. Nakamura, T. Kôno, and S. Funahashi, This Bulletin, **22**, 320 (1958).

2) T. J. Schoch, *J. Amer. Chem. Soc.*, **64**, 2957 (1942).
3) S. Lansky, M. Kooi, and T. J. Schoch, *ibid.*, **71**, 4066 (1949).
4) T. J. Schoch, and C. Williams, *ibid.*, **66**, 1232 (1944).
5) Z. Nikuni, K. Takaoka, and E. Fuwa, *J. Agr. Chem. Soc. Japan*, **26**, 218 (1952).
6) K. Takaoka and Z. Nikuni, *ibid.*, **26**, 186 (1952).

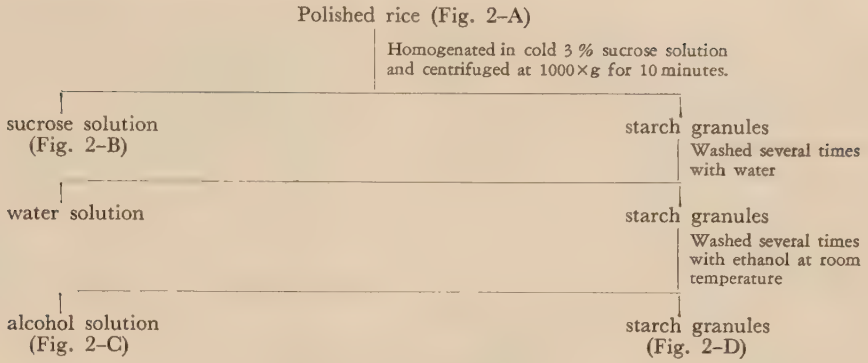

FIG. 1. Fractionation of Polished Rice.

and it is an interesting fact that lysolecithin which is present in the starch granules in rice and wheat has such an ability.

II. MATERIALS

a) Lysolecithin: Lysolecithin was prepared from rice grains after the method described by Iwata.[7]

b) Starch: Reagent grade, commercial potato starch was used.

c) Amylose and amylopectin: Amylose and amylopectin were prepared from the potato starch according to the method of Schoch[2,3], by using either butanol or amyl alcohol as the precipitating reagent.

III. GENERAL METHODS

The amount of lysolecithin was determined semiquantitatively, by paper chromatography after being extracted with hot ethanol, as it was described in the previous report[1]. Turbidity of the solution was measured as optical density using a Coleman nephelometer.

Spectra of iodine-starch complexes were measured with a Hitachi spectrophotometer. Samples were prepared by adding 0.05 ml. of 0.1 N iodine, dissolved in potassium iodine solution, to 10 ml. of 0.1 % starch solution.

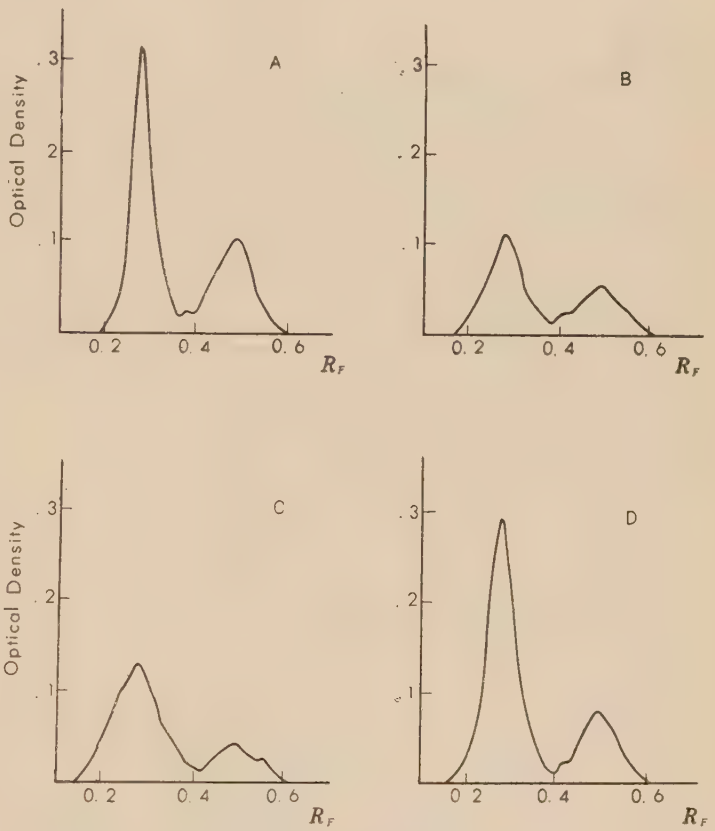

FIG. 2. Paper chromatograms of lysolecithin and lecithin in the fractions of polished rice. c.f. Fig. 1.

The amounts of phosphate were determined by the method of Nakamura.[8]

7) M. Iwata, *J. Agr. Chem. Soc. Japan*, **6**, 759 (1930).

8) M. Nakamura, *J. Agr. Chem. Soc. Japan*, **24**, 1 (1950).

Iodine titration of starch was performed according to Schoch et al,[2] by the dead-stop method using a micro-amperometric titration apparatus.[9]

IV. EXPERIMENTS AND RESULTS

A. Fractionation of polished rice homogenates. Polished rice grains were ground in 3 % sucrose solution, and fractionated by differential centrifugation. The procedure and the paper chromatograms of lecithin and lysolecithin in each fraction are indicated in Fig. 1 and Fig. 2.

As it is seen from these results, both lecithin and lysolecithin are associated with starch granules, and are slightly extracted with water or ethanol at room temperature.

The same results were obtained with non-glutinous wheat flour.

B. Interaction of lysolecithin with starch granules. The possibility of adsorption of lysolecithin by starch granules in the fractionation was investigated.

Twenty grams of rice starch granules were suspended in 50 ml. of water, to which 10 ml. of a 2% solution of lysolecithin added. Though lysolecithin adsorbed by the starch granules was almost quantitatively eluted by washing the granules with alcohol at room temperature, while the lysolecithin in nature was slightly extracted. (Fig. 3)

It may be concluded that lysolecithin is present in rice grains associated with starch granules, but is not merely adsorbed by the surface of the granules.

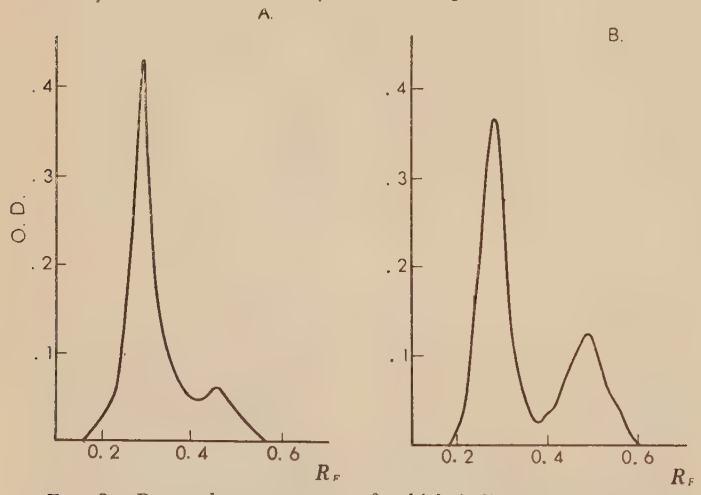

FIG. 3. Paper chromatograms of which indicate the interaction of lysolecithin with starch particles.

A: Lysolecithin adsorbed to starch particles in aqueous solution was eluted with ethanol for 30 minutes at room temperature. Parts of the natural lysolecithin and lecithin were also extracted as shown in Fig. 2-C.

B: The residue of A was further extracted with ethanol at 50° for 18 hours.

9) Y. Nagai, T. Kôno and S. Funahashi, This Bulletin, **21**, 121 (1957).

C. Interaction of lysolecithin with starch in aqueous solution.

1. With starch. Turbidity caused by the addition of lysolecithin in starch solution was measured.

Ten ml. of aqueous solution containing various amounts of lysolecithin was added to 10 ml. of hot 2 % starch solution; the mixture was boiled for a while, and then left at room temperature for several hours. The turbidities of the mixtures are shown in Fig. 4–b.

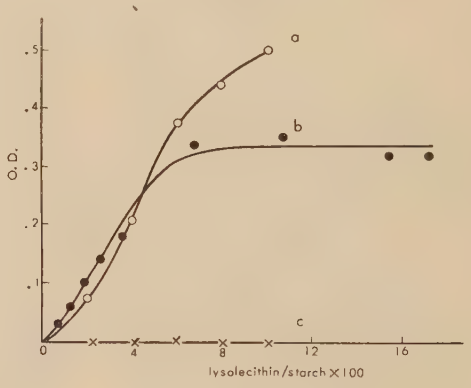

FIG. 4. Turbidities which were caused by the interaction of lysolecithin with a) ○—○ amylose, b) ●—● starch, and c) ×—× amylopectin in aqueous solution.

Starch solution without lysolecithin was taken as the control. The turbidity became saturated with 7.5 mg of lysolecithin per 100 mg of starch.

2. With amylose and amylopectin. The same experiments as described above were performed using amylose and amylopectin solutions. The results shown in Fig. 4–a and c. indicate that lysolecithin causes amylose to precipitate, but not in the case with amylopectin.

D. Separation of amylose and amylopectin by lysolecithin. One gram of potato starch was dissolved in 100 ml of water at 100°, and the solution was autoclaved at 15 lb. for 1 hours. To this solution, 100 mg of lysolecithin dissolved in 5 ml. of water was added, and the mixture was re-

fluxed for 1 hour. The solution was left at room temperature for several hours with constant stirring, and was kept in an ice-box over night. The resulting precipitate was centrifuged at 20,000×g for 1 hour to obtain the supernatant (s) and the precipitate (p). The supernatant (s) was concentrated in vacuo, and an equal volume of ethanol was added to precipitate the amylopectin-lysolecithin complex (Fraction B); the yield was 200 mg.

As the precipitate (p) contained a significant amount of the solution, it was further centrifuged at 40,000×g for 30 minutes, and the final precipitate was dissolved in 100 ml. of boiling water containing 50 mg of lysolecithin and reprecipitated by cooling, and the precipitate was centrifuged at 20,000×g for 1 hour. The reprecipitation was repeated once more, and the final precipitated was dried up by adding ethanol. The yield was 250 mg (Fraction A).

Fractions A and B were refluxed seven times in 10 ml. of ethanol to remove lysolecithin from the complex and to obtain amylose (Fraction A′) and amylopectin (Fraction B′).

E. Analyses of the fractions separated with lysolecithin.

1. Iodine-starch complex of the fractions. Absorption spectra of iodine-starch reaction of the fractions are shown in Figs. 5 and 6. The color of the iodine complex of both Fraction A and B were

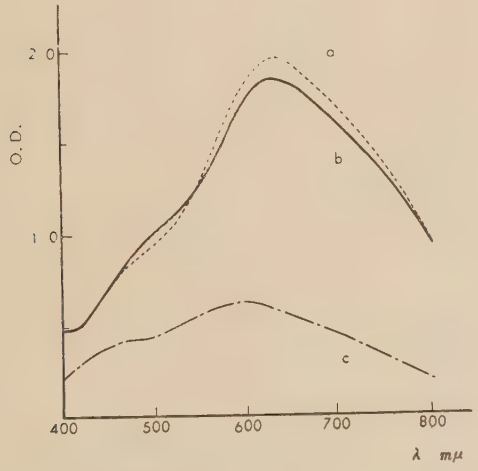

FIG. 5. Absorption spectra of iodine-starch complex of amylose fraction.
a) ----: standard amylose, b) ——: Fraction A′, and c) —·—: Fraction A.

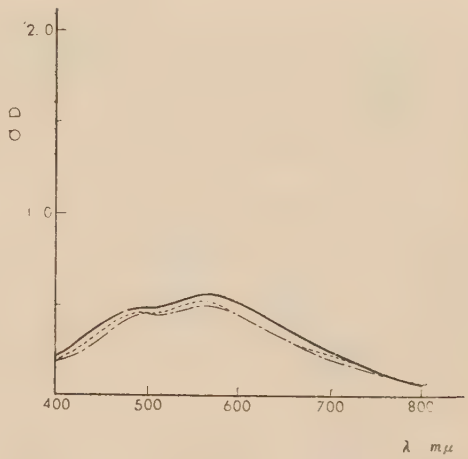

FIG. 6. Absorption spectra of iodine-starch complex of amylopectin fraction.
----: Standard amylopectin, ——: Fraction B′, and —·—: Fraction B.

red. The latter changed slightly, but the former changed to blue upon removal of lysolecithin, indicating that Fraction A′ was amylose and Fraction B′ was amylopectin.

2. Assay of phosphate and lysolecithin in the fraction. The amounts of phosphate in Fractions A and B were determined to estimate the amount of lysolecithin contained. The presence of lysolecithin in the alcohol extracts of Fractions A and B were proved by paper chromatography. The presence of choline-residue in the alcohol solutions was also proved by the use of Dragendorf's reagent. The results are summarized in Table I.

TABLE I
ASSAY OF PHOSPHATE, LYSOLECITHIN AND CHOLINE-RESIDUE IN THE FRACTIONS.

	Potato starch	Fraction A (Amylose)	Fraction B (Amylopectin)
P %	0.04	0.37	0.13
lysolecithin % calcd. from P content.	—	6.1	2.1
lysolecithin*	—	+	+
choline-residue**	—	+	+

* Identified by paper chromatography.
** Identified by Dragendorff's reagent.

It is calculated from the data in Table I, that one molecule of lysolecithin was included in 50 units of glucose in the case of Fraction A, amylose, and 150

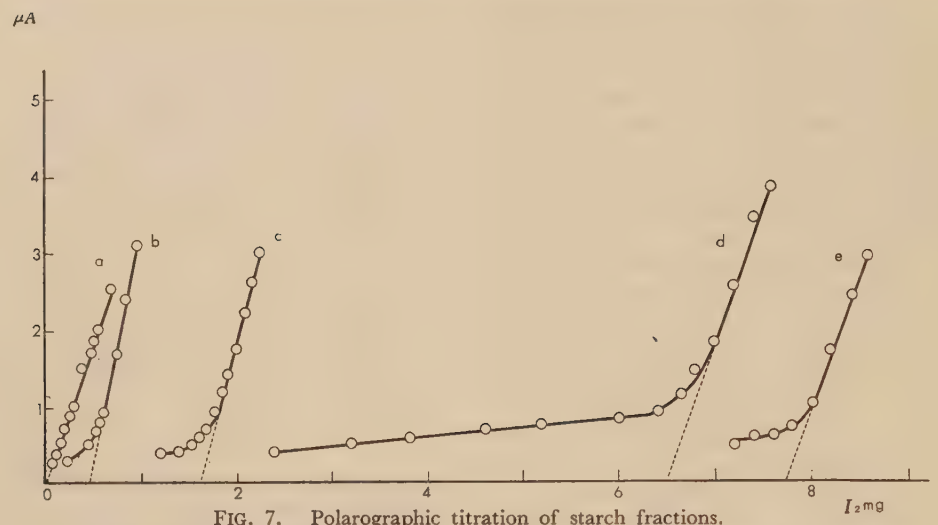

FIG. 7. Polarographic titration of starch fractions.

The indicated amount of each fraction was titrated with 0.200 wt. % of iodine solution. a) Amylopectin 40.5 mg, b) Fraction B′ 39.8 mg, c) Starch 38.2 mg, d) Fraction A′ 39.5 mg, e) Amylose 40.1 mg.

units of glucose in the case of Fraction B, amylopectin.

3. Iodine titration of the fractions. The length of the amylose chain of Fractions A and B were determined by iodine titration.[8] The data are illustrated in Fig. 7, together with the data of potato starch and of authentic specimens of amylose and amylopectin obtained by Schoch's method. These data also indicate that Fraction A is amylose and Fraction B is amylopectin, but a small amount of contamination of amylose in amylopectin fraction is observed.

4. Crystalline structures of lysolecthin-starch complexes. X-ray analyses of Fractions A and B were performed by Dr. Nikuni of Osaka University. The Fraction A gave a crystalline pattern close to that of butanol-amylose complex in X-ray analysis, but Fraction B was amorphous as in the case of amylopectin. Details will be published later.

V. DISCUSSION AND CONCLUSION

A. Lysolecithin is an element of starch granules in non-glutinous rice grains. The determination of lysolecithin on various fractions of rice grains indicates that lysolecithin is an element of starch granules. Although the added lysolecithin is adsorbed by starch granules from an aqueous solution, the adsorbed lysolecithin is easily extracted with ethanol at room temperature, while natural lysolecithin is slightly extractable at that temperature.

B. Complex formation of lysolecithin with starch. Although the nature of the binding of lysolecithin in starch granules is now being studied, the experiments in vitro give us some suggestions on that problem.

It was observed that lysolecithin causes amylose to precipitate, but does not amylopectin. The possibility that the precipitation might be caused by some impurities such as fatty acid is denied from the analyses of phosphate and the detection of lysolecithin in the precipitated fraction.

The red color of the iodine reaction of amylose-lysolecithin complex, which changes to blue by the removal of lysolecithin, indicates that lysolecithin is included in the helicoidal structure of amylose chain, as observed in the cases of butanol or amyl-alcohol complexes of amylose.

The iodine titration disclosed again that the fraction precipitated by lysolecithin is amylose. The same experiment, however, proved that a small amount of amylose was still contained in the amylopection fraction.

Thus, it is not very certain whether or not lysolecithin forms a soluble complex with amylopectin in aqueous solution, although the presence of lysolecithin was proved in Fraction B. The results of the X-ray analyses were also in accord with the above mentioned conclusion.

C. Possible role of lysolecithin in grains. The presence of lysolecithin would probably inhibit the enzymatic transformation of amylose into amylopectin in ripening seeds of rice, because lysolecithin causes amylose to precipitate from its solution. This assumption is consistent with the fact that lysolecithin is contained in non-glutinous rice grains in which amylose is present but not in glutinous rice grains in which amylose is absent. Enzymatic studies are now in progress.

VI. Acknowledgement

The authors express their sincere acknowledgement to Professor Ziro Nikuni of Osaka University for his kind suggestions concerning the possible role of lysolecithin in starch solution.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 330~334, 1958]

Relationship between Stereoisomerism and Biological Activity of Pyrethroids

Part II. Higher Homologues of Chrysanthemic Acid and Related Compounds

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Received May 24, 1958

(+)-*trans*-Homochrysanthemic acid, when boiled in dilute sulfuric acid, gives (+)-*trans*-ε-hydroxy-dihydrohomochrysanthemic acid, m.p. 176–7°, together with (+)-δ, δ-dimethyl-γ-*iso*-butenyl-δ-valerolactone. The formation of optically active lactone from (+)-*trans*-homochrysanthemic acid provides another cogent evidence for the structure of the lactone previously deduced on the racemic compound.

The Arndt-Eistert reaction of the homo-acids give further higher homologues such as (±)-, (+)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acids and (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid. Both *trans*-acids, in boiling dilute sulfuric acid, give the same (±)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone together with the corresponding hydroxy-acids, optically inactive and active, respectively.

Complete resolution of (±)-*trans*-homochrysanthemic acid and (±)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid was achieved by means of optically active α-phenylethylamine.

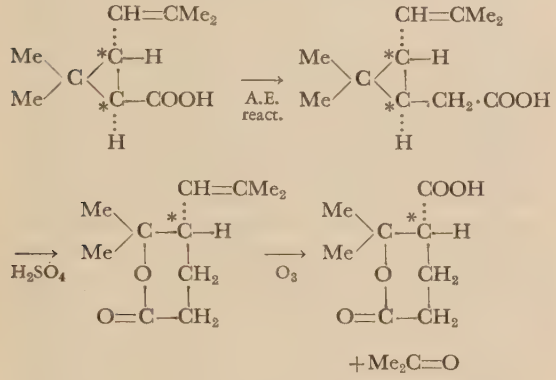
In the preceding paper[1] the authors reported the synthesis of homochrysanthemic acid and the related compounds.

Stereochemical consideration with an atomic model of *trans*-homochrysanthemic acid suggests that a hydrated compound containing the *cyclo*propane ring should also be possible, as was the case with *trans*-chrysanthemic acid which, under the same conditions, gave δ-hydroxy-dihydrochrysanthemic acid, exclusively.

In fact, the separative crystallization of the acidic residue from the neutral lactone fraction gave the expected (±)-*trans*-ε-hydroxy-dihydrohomochrysanthemic acid, m.p. 151–2°, in a poor yield. The hydroxy acid was reconverted into the parent homochrysanthemic acid by distillation *in vacuo* in the presence of *p*-toluenesulfonic acid. Optically active (+)-*trans*-homochrysanthemic acid was prepared by the Arndt-Eistert reaction of (+)-*trans*-chrysanthemic acid. (+)-*trans*-Homochrysanthemic acid, when treated with boiling dilute sulfuric acid in exactly the same manner as with the racemic acid, gave an optically active (+)-δ-lactone together with an optically active hydroxy-acid, (+)-*trans*-ε-hydroxy-dihydrohomochrysanthemic acid, m. p. 176–7°. The infra-red spectrum of the dextrorotatory lactone thus obtained was shown to be completely identical with that of the racemic lactone described in the preceding paper and on ozonolysis, the (+)-δ-lactone gave a laevorotatory lactonic acid, m.p. 196–7°, together with acetone (as 2,4-dinitrophenylhydrazone m.p. 126°) also consistent with the case of the rasemic compound. Therefore, the formation of the optically active δ-lactone from (+)-*trans*-homochrysanthemic acid should naturally necessitate the retention of the configuration of at least one of the asymmetric centres involved and, although the fission of *cyclo*propane ring at $C_{(1)}$—$C_{(2)}$ bond results in the

1) Y. Katsuda, T. Chikamoto and Y. Inouye, This Bulletin, **22**, 185 (1958).

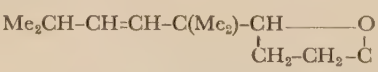
racemization of the asymmetric centre $C_{(1)}$, the other $C_{(3)}$ is expected to retain its activity during lactonization. Hence, the retention of optical activity during lactonization reasonably provides another cogent evidence for the structure of the lactone previously deduced for the racemic compound in Part 1.



Further extention of the carbon chain was effected by the Arndt-Eistert reaction of the homo-acids and (±)-, (+)-*trans*-β-(3-*iso*butenyl-2,2-dimethyl*cyclo*propane-1)-propionic acid and (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid were obtained. The structures of these higher homologous acids were evidenced chemically as well as spectrally in comparison with the parent compound.

A different situation arose in the lactonization of these higher homologues in boiling dilute sulfuric acids. Boiling (±)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid with 5% sulfuric acid for three hours sufficed to effect 82% conversion of the acid into a liquid lactone, b.p. 106–7°/3.5 mm.. The infra-red spectrum of this lactone indicated the bands characteristic for γ-lactone (1765 cm^{-1}) and ethylenic double bond (970 cm^{-1}, probably *trans*). The lactonization of the optically active acid under the same conditions occurred with complete loss of optical activity to give the racemic γ-lactone which was shown to be identical in every respect with that obtained from the racemic acid. The lactone absorbs bromine instantly, in contrast to pyrocin and to the δ-lactone obtained from homochrysanthemic acids, and was hydrogenated over a platinum catalyst to yield dihydrolactone, one molecule of hydrogen being taken up. The presence of one ethylenic double bond requires this lactone to be monocyclic, clearly indicating that a fundamental structural rearrangement of the parent acid occurred with fission of the *cyclo*propane ring at $C_{(1)}$—$C_{(3)}$ bond in contrast to the cases with chrysanthemic and homochrysanthemic acids.

The ozonization of the γ-lactone gave *iso*butyric acid, *iso*butyraldehyde (characterized by 2, 4-dinitrophenylhydrazone m.p. 181–2°) and C_8-lactonic acid (as *p*-phenylphenacylester m.p. 210–11°). These experimental data lead to the following structure for this lactone as:



In this case, consequently, the lactonization proceeded with the rupture of $C_{(1)}$—$C_{(3)}$ bond of *cyclo*propane ring, concomitant rearrangement of double bond $Me_2C{=}CH{-}CH{\rightarrow}Me_2CH{-}CH{=}CH{-}$ and simultaneous attack of the carboxyl-oxygen to the $C_{(1)}$ carbon atom to form γ-lactone. As by-products, (±)- and (+)-hydroxy acids (m.p. 97–8° and 119–120°) were also isolated in respective lactonization.

(±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid, upon undergoing the same reaction, gave a hydroxy acid m.p. 98–9° and a liquid lactone which was not identical with the above-mentioned γ-lactone. The structure has not yet been revealed and will be reported elsewhere in future.

Resolution of (±)-*trans*-homochrysanthemic acid by fractional crystallization of the quinine salt yielded the pure (+)-*trans* acid quinine salt, decomposed by hydrochloric acid to optically pure (+)-*trans* homochrysanthemic acid $[\alpha]_D^{16}$ +23.8°. Conversion of the impure (−)-*trans*-acid into the (−)-α-phenyl-

ethylamine salt and fractional crystallization yielded the pure (−)-*trans* acid (−)-α-phenylethylamine salt, decomposed to optically pure (−)-*trans*-homochrysanthemic acid $[\alpha]_D^{16}$ −23.3°.

By application of the same procedures, resolution of (±)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid yielded the pure (−)-*trans* acid quinine salt, decomposed to the oplically pure (−)-*trans*-acid $[\alpha]_D^{14}$ −40.4°. By conversion of the impure (+)-*trans*-acid into the (−)-α-phenylethylamine salt, the optically pure (+)-*trans* acid $[\alpha]_D^{14}$ +40.7° was isolated.

EXPERIMENTAL

Melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division of the Mitsui Laboratory, Kyoto University, to whom the authors' thanks are due. Infra-red spectra were determined with a Shimadzu double beam spectrophotometer. (Model AR-275).

(+)-*trans*-Homochrysanthemic acid By the same procedures as described by the authors previously[1], (+)-*trans*-chrysanthemoyl chloride (28.0 g.) and diazomethane (prepared from 120 g. of nitrosomethylurea) gave (+)-*trans*-homochrysanthemic acid, b.p. 105–6°/3 mm., n_D^{20} 1.4715, $[\alpha]_D^{20}$+23.3° (*c*, 3.00, ethanol), (17.6 g. 64% baseed on acid chloride), infra-red spectrum (cm^{-1}) 2970, 1720, 1450, 1235, 1165 and 1130. The equivalent weight by titration was 182.5 (Calcd. for $C_{10}H_{17}CO_2H$, 182.3). By the standard method, *p*-bromophenacyl-ester, m.p. 48–9° (*Anal.* Found C, 59.91; H, 6.10; Calcd. for $C_{19}H_{23}O_3Br$; C, 60.16; H, 6.11) was prepared.

(+)-δ, δ-Dimethyl-γ-*iso*butenyl-δ-valerolactone (+)-*trans*-Homochrysanthemic acid (3.0g.) was refluxed with 5% (v/v) sulfuric acid (65 ml.) for three hours. The reaction mixture was taken up in ether, washed three times with 5% sodium hydroxide, then with water, and the ether solution was dried over anhydrous sodium sulfate. Distillation then gave (+)-δ,δ-dimethyl-γ-*iso*butenyl-δ-valerolactone, b. p. 81–2°/2 mm., n_D^{20} 1.4585, $[\alpha]_D^{15}$ +37.2° (*c*, 1.80, ethanol), infra-red spectrum (cm^{-1}) 3000, 1775, 1470, 1380, 1280, 1110, 960 and 830. Yield 2.6 g.. The lactone gave a positive test with Denige's reagent and absorbed bromine slowly.

Ozonization (+)-δ, δ-Dimethyl-γ-*iso*butenyl-δ-valerolactone (1.0 g.) dissolved in chloroform (20 ml.) was ozonized at 0°. The solvent was evaporated under reduced pressure and the residual ozonide was decomposed with water (40 ml.) and left standing overnight. From the distillate, acetone was isolated as 2,4-dinitrophenylhydrazone m. p. 126° (mixed). On concentration of the remaining solution, (−)-δ, δ-dimethyl-γ-carboxy-δ-valerolactone (0.50 g.) m.p. 197–8°, $[\alpha]_D^{15}$−12.4° (*c*, 4.29, ethanol) *Anal.* Found C, 55.72; H, 7.01; Calcd. for $C_8H_{12}O_4$; C, 55.80; H, 7.03) equivalent weight by titration, found 174.2, Calcd. for $C_7H_{11}O_2$ (CO_2H) 172.2, infra-red spectrum (cm^{-1}) 3100, 2900, 1750, 1020, 985, 950 and 840.

(+)-*trans*-ε-Hydroxy-dihydrohomochrysanthemic acid (+)-*trans*-Homochrysanthemic acid (3.0 g.) was refluxed with 5% (v/v) sulfuric acid (65 ml.) for three hours. The reaction mixture was taken up in ether, washed with 5% sodium hydroxide, then the alkali solution was acidified and extracted with ether. On evaporation of the ether, extract, (+)-*trans*-ε-hydroxy-dihydrohomochrysanthemic acid crystallized in prisms (0.3 g.), m.p. 176–7° (ethyl acetate) $[\alpha]_D^{12}$ +24.0° (*c*, 1.00, ethanol), *Anal.* Found. C, 66.02; H, 10.09; Calcd. for $C_{11}H_{20}O_3$; C, 65.97; H, 10.07), equivalent weight by titration, found 200.8, Calcd. for $C_{10}H_{19}O$ (CO_2H) 200.3. The hydroxy-acid gave a positive test with Denige's reagent and a negative test with Nessler's reagent.

The hydroxy acid was distilled in the presence of *p*-toluene sulfonic acid under reduced pressure, when the hydroxy acid was dehydrated, to give (+)-*trans*-homochrysanthemic acid. Identity of the acid was provided by complete identity of infra-red spectra.

(±)-*trans*-ε-Hydroxydihydrohomochrysanthemic acid By the same procedures as described above, (±)-*trans*-homochrysanthemic acid (3.0 g.) gave (±)-*trans*-ε-hydroxy-dihydrohomochrysanthemic acid (0.3 g.) m. p. 151–2° (ethyl acetate), (*Anal.* found. C, 66.02; H, 10.24; Calcd. for $C_{11}H_{20}O_3$; C, 65.97; H, 10.07), equivalent weight by titration, Found 201.1. Calcd. for $C_{10}H_{19}O(CO_2H)$ 200.3. The hydroxy-acid gave a positive test with Denige's reagent and a negative test with Nessler's reagent. The hydroxy-acid was distilled in the presence of *p*-toluenesulfonic acid under reduced pressure to regenerate (±)-*trans*-homochrysanthemic acid.

(±)-*trans*-β-(3-*iso*Butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid By the same procedures as described by the authors previously,[1] (±)-*trans*-homochrysanthemoyl chloride (b.p. 96–7°/6 mm., 47 g.) and diazomethane (prepared from 150 g. of nitrosomethyl-

urea) gave (±)-*trans*-β-(3-*iso*butenyl-2,2-dimethyl*cyclo*propane-1)-propinic acid, b.p. 121–2°/5mm., n_D^{20} 1.4716, (30.4 g. 66 % based on acid chloride), infra-red spectrum (cm^{-1}), 2950, 1700, 1460, 1380, 1295, 930 and 850. This acid crystallized slowly, m. p. 50–1° (pet. ether). The equivalent weight by titration was 196.9 Calcd. for $C_{11}H_{19}CO_2H$ 196.3. By the standard method, *p*-bromophenacylester, m. p. 58–9° (*Anal.* Found, C, 61.03; H, 6.45; Calcd. for $C_{20}H_{25}O_3Br$; C, 61.07; H, 6.41) was prepared.

Hydrogenation (±)-*trans*-β-(3-*iso*Lutenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid (1.0 g.) in 20 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst in a shaking hydrogenation apparatus, and absorbed 121 ml. (at 16°) of hydrogen (equivalent to 1 mole), giving (±)-*trans*-β-(3-*iso*butyl-2,2-dimethyl-*cyclo*propane-1)-propionic acid, b.p. 125–6°/5 mm., n_D^{20} 1.4488, equivalent weight by titration, found 198.2, Calcd. for $C_{11}H_{21}CO_2H$ 198.3, infra-red spectrum, (cm^{-1}), 2950, 1700, 1465, 1380 and 1290. *p*-Bromophenacylester, prepared by the standard method, formed fine needles from ethanol, m. p. 53–4° (*Anal.* Found. C, 60.96; H, 6.71; Calcd. for $C_{20}H_{27}O_3Br$; C, 60.76; H, 6.88).

(+)-*trans*-β-(3-*iso*Butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid By the same procedures as in the case of racemic acid described above, (+)-*trans*-homochrysanthemoyl chloride (b.p. 78–80°/5mm., 22g.) diazomethane (prepared from 100 g. nitrosomethylurea) gave (+)-*trans*-β-(3-*iso*butenyl-2,2-dimethyl*cyclo*propane-1)-propionic acid, b.p. 125–6°/3 mm., n_D^{20} 1.4712, $[\alpha]_D^{20}$ +44.3° (*c*, 3.10, ethanol), (17.4 g. 81 % based on acid chloride), infra-red spectrum (cm^{-1}), 2950, 1700, 1460, 1380, 1295, 930 and 850. The equivalent weight by titration was 196.8. Calcd. for $C_{11}H_{19}CO_2H$, 196.3.

By the standard method, *p*-bromophenacylester, m.p. 35–6° (*Anal.* Found. C, 60.83; H, 6.37; Calcd. for $C_{20}H_{25}O_3Br$; C, 61.07; H, 6.41) was prepared.

Hydrogenation (+)-*trans*-β-(3-*iso*Butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid (1.0 g.) in 20 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst and 122 ml. (at 16°) of hydrogen (equivalent to 1 mole.) were taken up, giving the dihydro acid, b. p. 125–6°/5 mm., n_D^{20} 1.4489, $[\alpha]_D^{16}$ +4.9° (*c*, 3.07, ethanol), equivalent weight by titration, Found. 197.9 Calcd. for $C_{11}H_{21}CO_2H$ 198.3, infra-red spectrum (cm^{-1}) 2950, 1700, 1465, 1380, 1290 and 930. *p*-Bromophenacylester was prepared by the standard method, m. p. 46–7° (*Anal.* Found. C, 60.79; H, 6.73; Calcd. for $C_{20}H_{27}O_3Br$; C, 60.76; H, 6.88).

(±)-γ-(1′, 1′, 4′-Trimethyl-pent-2′-enyl)-butyrolactone

(a) From (±)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid. By the same procedures as in (+)-δ, δ-dimethyl-γ-*iso*butenyl-δ-valerolactone, (±)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid (5.0 g.) gave (±)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone, b. p. 106–7°/3.5 mm., n_D^{20} 1.4705, (4.1 g.), infra-red spectrum (cm^{-1}), 2950, 1765, 1740, 1460, 1388, 1280, 1175 and 970. The lactone absorbed bromine instantly.

As a by-product, (±)-hydroxyacid (0.5 g.) was isolated, m.p. 97–8° (pet. ether—ethyl acetate) (*Anal.* Found. C, 67.14; H, 10.23; Calcd. for $C_{12}H_{22}O_3$, C, 67.25; H, 10.35) equivalent weight by titration, found 215.2. Calcd. for $C_{11}H_{21}O(CO_2H)$ 214.3, infra-red spectrum (cm^{-1}) 2900, 1730, 980, 895, 860, 835 and 790.

(b) From (+)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid. By the same procedures as (a), (+)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid (4.5 g.) gave (±)-γ-(1′,1′,4′-trimethyl-pent-2′-enyl)-butyrolactone, b. p. 106–7°/3.5 mm., n_D^{20} 1.4716 (3.0 g.). This was identical with the lactone described above, that was shown by the identities of boiling point, refractive indices and infrared spectra, as well as the ozonolysis products (see below).

As a by-product in experiment (b), (+)-hydroxy acid (0.3 g.) was also isolated, m. p. 119–120° (pet. ether—ethyl acetate), $[\alpha]_D^{15}$ +17.0° (*c*, 3.00, ethanol), (*Anal.* Found. C, 67.32; H, 10.13; Calcd. for $C_{12}H_{22}O_3$; C, 67.25; H, 10.35) equivalent weight by titration, Found 213.8. Calcd. for $C_{11}H_{21}O(CO_2H)$ 214.3.

Hydrogenation (±)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone (0.5 g.) in 10 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst and absorbed 1 mole. equivalent of hydrogen (62 ml. at 18°), yielding the dihydrolactone, b. p. 129–130°/5 mm., n_D^{20} 1.4565, infra-red spectrum (cm^{-1}) 2950, 1760, 1730, 1470, 1388, 1290 and 1175.

Ozonization (±)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone (1.0 g.) dissolved in chloroform (20 ml.) was ozonized at 0°. The solvent was removed under reduced pressure and the residual ozonide was decomposed with water (40 ml.) and left standing overnight. From the distillate, *iso*butyric acid b. p. 154–5° and *iso*butylaldehyde (characterized by 2,4-dinitrophenylhydrazone m.p. 181–2°) were isolated. On concentration of the remaining solution, C_8-lactonic acid was isolated as *p*-phenylphenacylester m.p. 210–11° (*Anal.* Found C, 78.81; H, 6.31; Calcd. for $C_{22}H_{22}O_3$; C,

79.01 ; H, 6.63).

(±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl *cyclo* butane-1-acetic acid By the same procedures, (±)-*cis*-3-*iso*-butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acid chloride (b. p. 89–91°/7.5 mm., 29 g.) and diazomethane (prepared from 120 g. nitrosomethylurea) gave (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid, b.p. 113–4°/3.5 mm., n_D^{20} 1.4745, (17.6 g. 62 % based on acid chloride), infra-red spectrum (cm^{-1}), 2950, 1700, 1450, 1290, 1220, 1160, 935 and 845. The equivalent weight by titration was 196.8 Calcd. for $C_{11}H_{19}$-(CO_2H), 196.3.

Hydrogenation (±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl-*cyclo*butane-1-acetic acid (0.7 g.) in 20 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst and 87 ml. (at 18°) of hydrogen (equivalent to 1 mole) was taken up, giving the dihydroacid, b.p. 132–3°/5 mm., n_D^{20} 1.4541, equivalent weight by titration, found. 198.6 Calcd. for $C_{11}H_{21}CO_2H$ 198.3, infra-red spectrum (cm^{-1}), 2950, 1710, 1465, 1290, 1215, 1130 and 935.

Treatment with H_2SO_4 (±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid (5.0 g.) was refluxed with 5 % (v/v) sulfuric acid (65 ml.) for three hours, the lactone (4.0 g.) b. p. 102°/3.5 mm., n_D^{20} 1.4672, infra-red spectrum (cm^{-1}) 2950, 1780, 1720, 1470, 1390, 1280, 1100, 980 and 960, and the hydroxyacid (0.9 g.) m. p. 98–9°. (*Anal.* Found. C, 67.07; H, 10.49; Calcd. for $C_{12}H_{22}O_3$; C, 67.25 ; H, 10.35) were obtained. The lactone absorbed bromine instantly.

Resolution of (±)-*trans*-homochrysanthemic acid Quinine (32.4 g.) in warm ethanol (40 ml.) was added to a solution of (±)-*trans*-homochrysanthemic acid (18.2 g.) in ethanol (25 ml.) and the mixture was filtered and set aside overnight. Filtration then gave a first-fraction of salt (F 1 21.0 g.), m. p. 89–102°, $[\alpha]_D^{10}$ −113.3°. Addition of water (15 ml.) to the filtrate from F 1 gave a second-fraction (F 2, 19.5 g.) m. p. 76–81°, $[\alpha]_D^{15}$ −144.0°. Recrystallizations of F 1 from 3 : 2 aqueous ethanol, gave pure (+)-*trans*-homochrysanthemic acid quinine salt (12.9 g.), m.p. 117–119°, $[\alpha]_D^{12}$ −106.7°. Decomposition of this salt by shaking in ether with 2N-hydrochloric acid, washing with water, drying (Na_2SO_4) and evaporating gave pure (+)-*trans*-homochrysanthemic acid (4.3 g.), b. p. 114°/3.5 mm., $[\alpha]_D^{16}$+23.8° (*c*, 2.94, ethanol), n_D^{20} 1.4718.

Decomposition of F 2 gave impure (−)-*trans*-homochrysanthemic acid (7.0 g.). This acid (7.0 g.) and (−)-α-phenylethylamine (4.7 g., $[\alpha]_D^{11}$ −38.9°) were mixed in 3 : 2 aqueous ethanol (30 ml.) and set aside overnight. The first-fraction (10.0 g.) had m. p. 113–126°, $[\alpha]_D^{16}$ −18.7° and after recrystallizations from 3 : 2 aqueous ethanol, gave pure (−)-*trans*-homochrysanthemic acid (−)-α-phenylethylamine salt (5.0 g.), m.p. 129–130°, $[\alpha]_D^{14}$ −44.2°. Decomposition of this salt gave pure (−)-*trans*-homochrysanthemic acid (3.0 g.), b. p. 107°/3.0 mm., $[\alpha]_D^{16}$ −23.3° (*c*, 3.00, ethanol), n_D^{20} 1.4718.

Resolution of (±)-*trans*-β-(3-*iso* Butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid Quinine (32.4 g.) in ethanol (40ml.) was added to a solution of (±)-*trans*-β-(3-*iso* butenyl-2, 2-dimethyl *cyclo* propane-1)-propionic acid (19.6 g.) in ethanol (25 ml.) and the mixture was filtered and set aside overnight. Filtration then gave a first-fraction of salt (F 1 20.0 g.), m.p. 84–94°, $[\alpha]_D^{14}$ −124.0°. Addition of water (15 ml.) to the filtration from F 1 gave a second-fraction (F2 20.0 g.), m.p. 76–88°, $[\alpha]_D^{19}$ −106.0°.

By the same procedures as described above, F1 gave pure (−)-*trans*-β-(3-*iso*butenyl-2,2-dimethyl*cyclo*propane-1)-propionic acid (3.6 g.), b.p. 118–9°/4.5 mm., $[\alpha]_D^{14}$ −40.4° (*c*, 3.22, ethanol), n_D^{20} 1.4715.

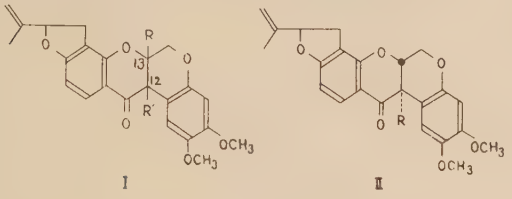
Also F 2, gave pure (+)-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionic acid (2.7 g.), b.p. 118–9°/4.5 mm., $[\alpha]_D^{14}$ +40.7° (*c*, 3.10, ethanol), n_D^{20} 1.4714.

Acknowledgements The authors are indebted to Prof. S. Takei, Prof. M. Ohno and Dr. Y. Inoue of Kyoto University for their advice and also to Mr. K. Uyeyama, President of this Company, for his encouragement in the course of this work.

Synthesen und Konfigurationsermittlung in der Rotenoid-Reihe, VI.

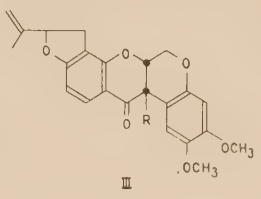
Die Struktur und Konfiguration von Rotenolonen

Rotenolon-I und -II wurden von Takei und Mitarbeitern[1] bei der Oxydation von Rotenon mit atmosphärischem Sauerstoff in schwach alkalischer Lösung erhalten, und die in der Formel (I) (Rotenolon-I: R=H, R'=OH; Rotenolon-II: R=OH, R'=H) angegebene Struktur wurde vorgeschlagen, die nachher von LaForge und Mitarbeiten[2] weitgehend diskutiert wurde. Diejenigen amerikanischen Autoren[2] haben abgeleitet, dass es sich um Stereoisomere handelt, dessen Oxygruppe möglicherweise auf der C_{12}-Stelle liegt.

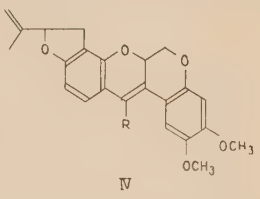


I

II



III



IV

In der vorliegenden Zuschrift möchten wir sowohl die Struktur als auch die Konfiguration beider Rotenolone mitteilen. UV-Spektren von Rotenon, Rotenolon-II und Acetylrotenon (IV, R=OCOCH$_3$) in Äthanol sind:

Rotenon	λ_{max}	292.5 mμ (ε 19700)
Rotenolon-II	λ_{max}	292.5 mμ (ε 20300)
Acetylrotenon	λ_{max}	355 mμ (ε 35400), 371 mμ (ε 30200)

In 0.8% alkoholischer Kalilauge weist Rotenon ausser dem oben beschriebenen Maximum die neuen Absorptionen von λ_{max} 354mμ (ε 10360), 371 mμ (ε 9530) auf, welche sich offensichtlich der Anwesenheit der entsprechenden Enolform (IV, R=OH) zuschreiben lassen. UV-Spektrum von Rotenolon-II zeigt im Gegensatz zum Rotenon kein Maximum in längeren Wellen. Daher liegt es nahe, dass Rotenolon-II kein enolisierbares Wasserstoffatom an C_{13} trägt. Bei der Oxydation von Rotenon mit Wasserstoffperoxyd in Gegenwart von Kalilauge haben LaForge und Mitarbb.[2] kein kristallines Produkt erhalten. Behandelt man dagegen eine Aceton-lösung von Rotenon mit Wasserstoffperoxyd unter dem Einfluss von Natronlauge zur Entfärbung der stark hellgelben Lösung[3], so lässt sich das Rotenolon-II aus der neutralen Fraktion isolieren. Schmp. 215–7° (unkorr.) weist bei Mischung mit einer authentischer Probe keine Erniedrigung auf. Analysenzahlen: C 67.4, H 5.5 (Ber. für $C_{23}H_{22}O_7$: C 67.3, H 5.4). Dieses Ergebnis bestätigt ferner, dass die neu verknüpfte Oxygruppe die C_{12}-Stelle besitzt wie in (II) bzw. (III) angegeben. Es hat sich gezeight[2], dass Methylrotenolon-II durch Behandlung mit methanolischer Schwefelsäure in Methylrotenolon-I übergeht. Daher muss das letztere die mehr stabilere Konfiguration (II, R=OCH$_3$) als das erste (III, R=OCH$_3$) besitzen. Da bei der Methylierung von Rotenolonen zu den angehörigen Methylrotenolonen freilich keine Umkehrung entsteht, können wir nun dem Rotenolon-I die Struktur (II, R=OH) und dem Rotenolon-II die alternative Struktur (III, R=OH) zuordnen. Dem natürlichen Rotenon kommt die trans-Konfiguration (II, R=H) zu[4] (s. auch die nachfolgende Mitteil-

1) S. Takei, S. Miyajima und M. Ohno, *Ber.* **66**, 479 (1933).
2) F. B. LaForge, H. L. Haller, *J. Am. Chem. Soc.*, **56**, 1620 (1934).
3) Diese Farbe stammt von Enolform Rotenons.
4) M. Miyano und M. Matsui, Dieses Bulletin, **22**, 128 (1958).

ung). Kürzlich hat Fukami[5],[6] entdeckt, dass Rotenon L-Glutaminsäure-Oxydase einiger Insekten verstört. In einem Fall, wo das Rotenon die Aktivität betreffendes Enzyms 84% hemmt, hemmt das Rotenolon-I 75% und Rotenolon-II nur 6% derselben. Dieses Resultat steht mit der oben beschriebenen Konfiguration im Einklang und lädt uns zu einer Hypothese ein, Rotenoide mit trans-Konfiguration mehr giftiger als das alternative Isomere. Aus Analogiegrü nden glauben wir, Deguelinol-I[7] (Tephrosin[8]) und -II[7] (iso-Tephrosin[9]) gegen Deguelin so angeordnet zu sein wie Rotenolon-I und -II gegen Rotenon.

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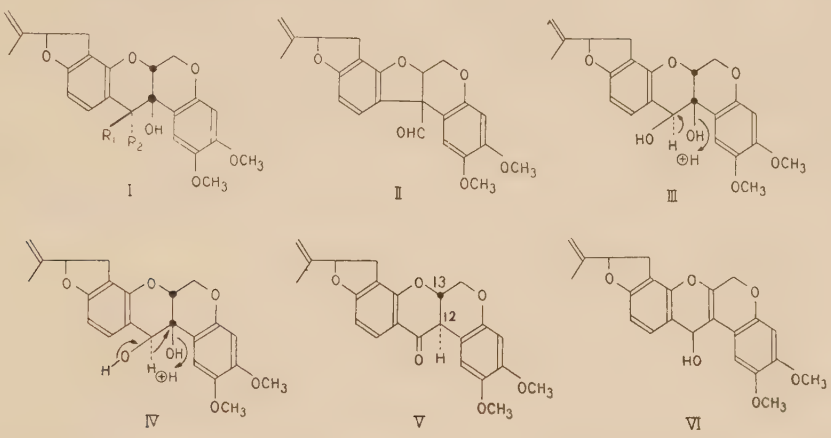
5) J. Fukami, *Botyu-Kagaku*, **21**, 122 (1956).
6) J. Fukami und Ch. Tomizawa, *ebenda*, **23**, 1 (1958).
7) S. Takei, S. Miyajima und M. Ohno, *Ber.*, **66**, 1826 (1933).
8) E. P. Clark, *J. Am. Chem. Soc.*, **53**, 729 (1931).
9) F. P. Clark und H. V. Claborn, *J. Am. Chem. Soc.*, **54**, 4454 (1934).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 5, p. 337~338, 1958]

Synthesen und Konfigurationsermittlung in der Rotenoid-Reihe, VII.

Eine Herstellung des natürlichen Rotenons aus Rotenolon-II; Ein weiterer Beweis für die Struktur von Rotenolon-II; Die Konfiguration des natürlichen Rotenons

Zwei erwartete Alkohole werden bei der Reduktion von Rotenolon-II mit Natriumborhydrid erhalten, welche wir als Rotenolol-α (I, R_1=OH, R_2=H) und Rotenolol-β (I, R_1=H, R_2=OH) bezeichnen. Rotenolol-α: Schmp. 251–2°, Analysenzahlen C 66.9, H 5.9 (Ber. für $C_{23}H_{24}O_7$ C 66.9, H 5.9), Rotenolol-β: Schmp. 147–8°, Analysenzahlen C 67.1, H 5.8 (Ber. für $C_{23}H_{24}O_7$ C 66.9, H 5.9) IR-Spektren stehen mit den Strukturen im Einklang. Behandelt man Rotenolol-α in Dioxan-Alkohol-Wasser mit Salzsäure, so wird das Rotenon in mässiger Ausbeute erhalten. Schmp. ist 160–1°, der Mischschmp. mit dem natürlichen Rotenon zeigt keine Depression. Das IR-Spektrum in Nujol Paste stimmt mit demjenigen natürlicher Probe ganz überein. Analysenzahlen: C 69.9, H 5.8 (Ber. für $C_{23}H_{22}O_6$: C 70.0, H 5.6). Die Struktur von Rotenolon-II wird also chemisch endgültig festgestellt.[1]) Bei der Säureeinwirkung liefert Rotenolol-β nicht das Rotenon sondern ein öliges Produkt nach chromatographicher Reinigung, dessen IR-Spektrum C=O Band an 5.77 μ besitzt. Dieses hellgelbe Öl leicht liefert 2,4-Dinitrophenylhydrazon. Obgleich wir weder den kristallinen Aldehyd noch das reine Dinitrophenylhydrazon erhalten, kommt dieser Verbindung die in der Formel (II) angegebene Struktur zu. Diese Befunde werden verständlich, wenn Rotenolol-β die Struktur (I, R_1=H, R_2=OH) hat.



Für die Chemismus der Umwandlung des Rotenolol-α kommen zwei Möglichkeiten in Betracht, von denen besteht der eine (III) darin, dass Rotenolol über die Enolform ins stabilere *trans*-Rotenon übergeht, während der zweite (IV) die Umkehrung der Konfiguration von C_{12} verursachen muss, d.h. das *trans*-Rotenon entsteht. Zusammen mit anderen Beweisen[2),3)] liegt es nabe, dass das auf Fische

1) Dehydrorotenol (VI) geht durch die Einwirkung von Salzsäure ins Rotenon nicht über.[3])

2) R. S. Cahn, R. F. Phipers und J. J. Boam, *J. Chem. Soc.*, **1938**, 513.

3) M. Miyano und M. Matsui, Diese Bulletin, **22**, 128 (1958).

und Insekten giftig wirkende natürliche Rotenon die trans (threo)-Konfiguration (V) besitzt.

Herren Professor Y. Sumiki, Hrn. Professor R. Yamamoto und Hrn. Dr. S. Tamura danken wir für die Unterstützung und Förderung dieser Arbeit. Hrn. Dr. M. Nakajima und seinen Kollegen zu Universität Kyôto danken wir herzlich für freundliche Diskussion und Überlassung einiger Proben. Wir sind Hrn. Dr. Y. Chen, Universität Taipei, Formosa, Hrn. Y. Ishii, Tôa-Nôyaku A.G., und Hrn. T. Yamamoto, Kakenkôgyô A.G. für ihr Interesse und bereitwillige Überlassung von Rotenon zu bestem Dank verpflichtet. Wir sagen vielen Dank Hrn. K. Aizawa für Aufnahme von IR-Spektren, Fr. K. Satô, Frl. M. Suzuki, und Frl. I. Isobe für Mikroanalysen.

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α-Amino Isobutyric Acid as a Constituent Amino Acid of Protein

(Studies on Muscle Proteins. Part 14)

Sir:

α-Amino isobutyric acid as a constituent amino acid of protein was at first reported by T. Yabuta[1] and his collabolates in 1938 and isolated as 5,5′-dimethylhydantoin in the dry distillate of pupae of silkworm.

Thereafter, few reports have appeared concerning this fact until Y. Oshima[2] et al. in 1953 recognized the presence of this amino acid in the acid-hydrolysate of casein by paper chromatography.

M. Kandatsu[3], one of the authors, confirmed the production of acetone from the acid-hydrolysate of rabbit muscle proteins by the oxidation of hypochlorite and suggested its presence in the protein, because α-amino isobutyric acid produces acetone exclusively by the oxidation of hypochlorite among known amino acids.

Continuing our investigations to clear up its presence in muscle protein, we could now isolate this amino acid from both of the acid- and pepsin-hydrolysates of horse muscle proteins which were prepared from the hind leg.

A sample of 700 g of horse muscle proteins was hydrolysed by 10-fold 20% HCl solution under reflux for 20 hrs., then humin was filtered off, and concentration in vacuum repeated to remove as much Cl' as possible. The hydrolysate was converted to copper salts with $CuCO_3$ and $Cu(OH)_2$ by the usual method.

The hydrolysate decomposed by enzyme was prepared from 100 g of muscle proteins by the usual method with concentrated pepsin at pH 2.0–2.2 and 39°C, for 40 hrs. and after adding 3% CCl_3COOH solution, the coagulants were filtered off. With the filtrate, copper salts were formed as described above.

The copper salts were fractionated with water and dry methanol, and the water soluble and methanol insoluble fractions were collected.

	Acid-hydrolysate	Pepsin-hydrolysate	Calculated for $C_4H_9NO_2$
C (%)	46.71	46.06	46.60
H	8.95	8.07	8.74
N	13.59	13.86	13.60
Sublimating point (°C)	275–	281–	280
R_F (phenol)	0.58		0.58
Picrate			
m.p. (°C)	194	194	193–4
C	36.61		36.14
H	4.11		3.61
N	16.82		16.87
Picrolonate			
m.p.	232–3		236
C	45.84		45.78
H	4.22		4.63
N	19.44		19.07

After removal of copper by H_2S, diamino and dicarboxylic acids were removed by

1) T. Yabuta et al, *Bulletin of the Institute of Physical and Chemical Research*, **17**, 1241 (1938).
2) Y. Oshima et al, *J. Agr. Chem. Soc. Japan.*, **27**, 102 (1953).
3) M. Kandatsu, *J. Agr. Chem. Soc. Japan*, **22**, 7 (1948).

phosphotungstic acid and $Ba(OH)_2$-ethanol, respectively. The filtrate was again treated as mentioned above to form copper salts, then the water soluble and methanol insoluble fractions were collected. After the removal of copper, the mono-amino mono-carboxylic fraction was concentrated under vacuum and treated by silica-gel chromatography with phenol : H_2O (8 : 2). The elute which contained this amino acid only was shaken with ether to remove phenol and added with absolute ethanol to crystallize the amino acid.

By repetition of recrystallization with ethanol, about 140 mg of white prismy crystalline was obtained. (Including about 20 mg from enzyme hydrolysate)

The properties of this crystalline are summerized in the table.

The appearance of sublimation, results of elementary analysis, are consistent with thoretical values and m.p., elementary composition of picrate and picrolonate and also infra-red absorption spectrum of this amino acid are identical with a synthetic sample.

Accordingly, it is confirmed by us that α-amino isobutyric acid is one of the constituent amino acids of the muscle protein.

The distribution of this amino acid in various proteins and its metabolism in higher animals is now under study.

Details of this experiment will appear in this journal.

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Studies on Continuous Fermention. Part VIII. Mixing Condition of Input and Fermenting Mashes in Continuous Fermentor. (p. *491~495*)

By Kiyomoto UEDA and Shinya TEZUKA

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The purpose of this study is to find the economical mixing condition of input and fermenting mashes in continuous-flow stirred fermentor. The experiments were carried out chiefly using the jar fermentor equiped with a external circulating pump, and the mixing conditions were examined by means of continuous purging process. It was found that the ratio of volumetric recirculation rate (Q) to volumetric throughput rate (F) is the most effective factor for the mixing of mashes. Using the jar fermentor, the perfect mixing was nearly obtained if the recycle rate Q/F is 6 or larger, and was assumed that the economical mixing condition of continuous alcoholic fermentation is obtained if recycle rate is about 6.

On the Microflora of the Rice found in Burma (Studies on the Microorganisms of Cereal Grains. Part II) (p. *496~500*)

By Hiroshi IIZUKA

(*Institute of Applied Microbiology, University of Tokyo*)

In investigating the causes of deterioration of cereal grains under the influences of microorganisms, the author has held the opinion that the most important method of approach is to trace the movements of microflora in an ecological system termed cereal grains. Studies on the cause of the deteriorated rice produced in Bruma, have been carried out in the same view.

In regard of the movements of microflora during the storage period of unhusked rice in Burma, new crop rice gives very few *Aspergillus* and *Penicillium*, and many so-called yellow *Pseudomonas*, however, in normal rice, after storage during the rainy season in the form of unhusked rice, the number of *Aspergillus*, *Penicillium* and *Streptomyces* increase.

There is some rice of which deterioration can be recognized by visual inspection. The common feature of this deteriorated rice, is that it contains considerably larger amounts of *Aspergillus*, *Penicillium* and *Streptomyces* than normal rice. Up to the present, no attention has been directed to *Streptomyces* found in rice. However, from the authors investigation many of these were found to be present in almost every specimen of normal rice.

It was further found that some of these *Streptomyces* possess a considerably poisonous function when mice are fed with food containing rice on which these strains are grown on.

Studies on the Effects of Some Physical Conditions on the Submerged Mold Culture. Part I. The Mycelial Growth of *Asp. niger* in the Shaking Culture, and the Effect of the Inoculum Size on its Pellet Formation. (p. *501~506*)

By Joji TAKAHASHI, Koichi YAMADA and Toshinobu ASAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

In the shaking cultures of molds, it is known that the mycelium may grow into homogeneous, filamentous suspensions (filamentous-type growth), or may develop into a from of globose colonies (pellet-type growth).

Many culture conditions may be considered to have some influences on the type and history of mycelial growth.

In this paper, the developmental history of *Asp. niger* NRRL 337 in two kinds of media (peptone-glucose and synthetic), will be presented at various stages of growth from the beginning of spore germination up to formation of spherical pellets. And, the effect of the size of spore inoculation on the type of mycelial growth of *Asp. niger* is also discussed in comparison with that of *Pen. chrysogenum* which was reported by Chain et al. in 1952.

Research on the Sugar Rifining. XVIII. On the Reaction between Amino Acids and Sugars. Part I. (p. *506~510*)

By Toshihide SHIMIZU and Shigeo SAKAI

(*The Hyogo University of Agriculture*)

As a fundamental study of colouring matters in raw

sugar solutions, the reaction between sugars (glucose, fructose) and amino acids (glutamic acid, aspartic acid, alanine) in cane sugar juice was investigated. The browning reaction was examined under the nearer conditions of the carbonation process. The products formed in this reaction were measured by a Beckman spectrophotometer (Model UV) and paper chromatography.

(1) The reactive solutions between sugars and amino acids were characterized by the absorption maximum at 265 mμ in the ultraviolet region. The positive substances to the ninhydrin reaction were separated from the solutions by paper chromatography, and characteristic absorption peaks at about 247 mμ and 287 mμ were observed. From the results, it may be supposed that ammonia produced from amino acids reacted the decomposition products of sugars and formed the N-glucosides. Moreover, fructose appeared to be more sensitive than glucose.

(2) A lowering of pH occurred with the progress of reaction and in the case of fructose it was more rapid than in the case of glucose.

(3) On the examination of the catalytic action for this reaction by inorganic salts, Na_2SO_4 and $Fe_2(SO_4)_3$ showed a negative action, and also, NaCl and $FeCl_2$ showed the same action, except the reaction between sugars and aspartic acid.

Studies on the Cellulose-decomposing Bacteria in the Rumen. Part III. Methods to form Colonies of these Bacteria without Accumulating Culture.

(p. *510～512*)

By Akira AKASHI

(*Department of Zootechnical Science, Faculty of Agriculture, Kyushu University*)

In previous reports (1, 2), a method to isolate the cellulose-decomposing bacteria in a short period of time by the use of Omeliansky's salts medium containing galactose, fuchsin and agar except cellulose as the carbon source has been reported. In this paper, a new technique as follows is presented: isolation and cultivation without the accumulation of culture by using the devised medium containing 0.5% cellobiose, 0.05% galactose, Omeliansky's salts and agar, and, by this way some strains of these bacteria were successfully isolated, and so, it is possible to determine the numerical relation to other bacteria in the rumen.

On the Bitter Substrated separated from Alcohol Distillation of Sweet Potato Mash.

(p. *513～514*)

By Tomotsune TAIRA and Yayako FUKAGAWA

(*Kyoto Women's University*)

An oily substance deposit was of ten observed in the distilled alcohol when alcohol was prepared from sweet potato mash. A bitter substance, ipomeamarone produced by the infection of *Ceratostomella fimbriata* Fujikuro on sweet potato, was studied in detail by Kubota (J. Chem. Soc. Japan, Vol. 73–78 (1952–1957)) and this was confirmed to be α-methyl, α-(ketoisohexyl)-α'-furyltetrahydrofuran. The oily substance separated from alcohol distillation of a sweet potato mash was supposed to be ipomeamarone, but is still unproved. The authors studied the oily substance collected by Ko-ei Co. Osaka, through the fractionation of Widmer's column. The main fraction was identified as ipomeamarone by its physical constants, elementary analysis and its semicarbazone (m.p. 132°C). In addition, there was a small amount of β-furoic acid (m.p. 123°), was found and presamed as decomposition product of ipomeamarone. The authors observed that even though when the alcohol was distilled with a small amount of ipomeamarone, the distillate gave no bitter taste; it was toxic to mice, $LD_{50}=230$, and results of the antimicrobial spectrum by the agar dilution method were as follows:

Organism	Minimum inhibitory concentration γ/cc
Aspergills oryzae	500
Asp. niger	500
Penicillium chrysogenum	350
Pen. notatum	500
Mucor mucedo	500
Rhyzopus nigricans	500
Saccharomysces cerevisiae	1000
Hansenula anomala	1000
Zygosaccharomyces soja	200
Torula rubra	200
Escherichia coli	<500
Proteus vulgaris	<500
Staphylococcus aureus	50
Bacillus subtilis	30

Steroids and Microbes. Part V. 11α-Hydroxylation of Steroids by *Metarrhizium anisopliae*.

(p. *515～517*)

By Yuichiro KUROSAWA

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The earliest record of the microbiological transfor-

mation of a steroid is that of Mamoli and Vercellone, dealing with the reduction of a ring ketone with yeast. During the last few years, interest in this field has been reactivated.

Particularly, regarding this problem Hench et al. demonstrated the dramatic effect of cortisone and hydrocortisone in the medical treatment of rheumatoid arthritis. A major obstacle encountered in this effort was the introduction of the biologically necessary oxygen at carbon No. 11.

For this purpose, microbiological methods have proved to be outstandingly successful. In 1952, Peterson et al. made an important discovery of hydroxylation in the 11α-position, using the molds of *Mucoraceae*.

The author has attempted the transformation of 3,5-cyclo-6β-hydroxy-pregnan-20-one and pregnenolone by *Metarrhizium anisopliae*.

As a result, it was found that these compounds are oxidized to 11α-hydroxylated compounds.

Its α-orientation was followed with known allopregnan-3β, 11α, 20β-triol m.p. 248–9° and allopregnane-3, 11, 20-trione m.p. 212–4°.

On the Types of the Acidic Amino Acid Recidues in the Vegetable Proteins as Starting Materials of Sho-yu. (p. *518~521*)

By Yasuhiro KONDO, Ryohei AOKI and Tetsuo OGAWA

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The types of ω-carboxyl groups of acidic amino acid residues of soy-bean gluterin and wheat gluten, i.e. the typical protein source of sho-yu, are investigated by means of AKABORI's hydrazinolysis-DNP-method[1], from which it was elucidated that most of these residues are of the amide type and/or branched type. The results obtained are illustrated as follows:

Types of ω-carboxyl groups

$$\frac{\text{amide type} + \text{branched type}}{\text{total glutamic or aspartic residue}} (\%)$$

	gluerin	gluten
glutamic residue	73	94
aspartic residue	78	74

1) S. Akabori, K. Ohno, T. Ikenaka, Y. Okada, H. Hanafusa, I. Haruna, A. Tsugita, K. Sugae and T. Matsushima, *Bull. Chem. Soc. Jap.* **29**, 507 (1956).

Studies on *Wasabi*. Part IV. Determination of Sinigrin. (p. *521~525*)

By Zenji NAGASHIMA, Masaaki UCHIYAMA and Yasushi UTSUGI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Shizuoka*)

The authors' attension has been directed to sinigrin, the mustard oil glucoside of black mustard and *wasabi*, which is said to reduce potassium ferricyanide quantitatively. By utilization of this characteristic, a method for the determination of sinigrin was devised.

By applying this method to plant extracts, interfering substances such as sugars were separated by application of ion-exchange resins.

By means of this method, sinigrin in the root stalk, leaf stalk and leaf of *wasabi* was determined.

The Formation of Amino Acids in Ki-Moto. Part II. On the Formation of Amino Acid Precursor in Ki-Moto and Experimental Trial of Yamahai-Moto. (p. *526~529*)

By Hiroichi AKIYAMA

(*Brewing Experiment Station, Tax Administration Agency*)

In respect to the reason for rich in amino acids in Ki-moto, the classical yeast inoculum, the author presumed that "amino acid precursor", an intermediate of proteolysis of steamed rice protein, was formed in early stage of moto making process.

As previous paper were reported, the precursor was water insoluble, 0.5% lactic acid solution soluble and at the acidic condition hydrolyzed easily to lower molecular peptides and amino acids. The precursor formed at low temperature was more easily decomposed than formed at high temperature over 40°C.

According to these results the author made moto rich in amino acids in small scale adding lactic acid in parts instead of lactic acid fermentation as Ki-moto.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part VII. On the so-called "Acid-forming Fermentation" in Acetone-Butanol Fermentation. (2) (p. *530~534*)

By Shigeru NEMOTO

(*Gôdô-Shusei Inc.*)

The author has investigated the causes of the abnormal fermentation reported in the previous paper.

Inoculation of the soured mash to corn mash before inoculating butanol bacillus, did not evolute gas and acids were not formed. These phenomena were similar to acertain type of the abnormal fermentation observed in our factory. But, on the contrary, the addition of the soured mash after butanol bacillus, the acid-forming fermentation reappeared. The author then isolated a

strain of acid-producing organism from the soured mash. This organism produced lactic acid, ethyl alcohol and other volatile acids and was found in the cooling water used in the factory.

By mixed cultures of the butanol bacillus and acid-producing bacillus, acid-forming fermentations were carried out whether the latter might be inoculated before or after the former.

After investigating the effect of cooling water on the butanol bacillus, the same results as in the case of the soured mash were obtained. The three types of the abnormal fermentations that occurred in the factory reappeared by varying the inoculating time of cooling water or the soured mash.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part VIII. On the so-called "Acid-forming Fermentation" in Acetone-Butanol Fermentation. (3) (p. *534~537*)

By Shigeru NEMOTO
(*Gôdô-Shusei Inc.*)

Microbiological characteristics of the isolated contaminant from soured mash and cooling water used in the factory, have been studied. According to Bergey's Manual of Determinative Bacteriology (sixth edition, 1948), the bacillus was identified as a variety of *Lactobacillus buchneri*.

As this organism is widely distributed in factory water, so the author attempted to prevent the mash from contact with water. Consequently abnormal fermentations were ceased. Some considerations in concern of these phenomena are given.

Studies on the Effects of Dextran Manufactured in Japan on the Physiological Function. Part II. Effects of Dextran on Blood Viscosity, Coagultion and its Toxity, Antigenicity. (p. *537~541*)

By Takehiko KAWANO
(*Research Laboratory, The Blood Plasma Corporation of Japan*)

Following the previous paper which the effects of Japanese dextran on the physiological function of erythrocytes were reported, the author has continued studies on the effects of dextran on the physiological function, through blood viscosity, coagulation and toxity, antigenicity of the dextran. Especially, the effect of dextran upon blood-clotting time is reported in detail, compared with other infusion fluids.

The author comes to the following conclusion.

(1) Blood viscosity is hardly influenced upon admixture with Japanese dextran.

(2) Dextran shortens blood-clotting time, when it is contained in low concentrations in blood and delays it in that of the high concentrations, but the delay is in general not remarkable until the concentration of the dextran solution in blood attains 50 per cent.

(3) In the existence of such high concentrations of dextran that delays-blood clotting time, a certain change of blood-clotting aspects is observed.

(4) Japanese dextran has neither toxity, nor antigenicity as far as the author's studies of its effects on rabbits and guinea pigs is concerned.

Studies on the Effects of Dextran Manufactured in Japan on the Physiological Function. Part III. On the Replacement Capacity of Dextran. (p. *542~544*)

By Takehiko KAWANO
(*Research Laboratory, The Blood Plasma Corporation of Japan*)

The author reports results of his experiment replacing blood with Japanese dextran solution in order to finally determine the effects of dextran as a plasma volume expander, in comparison with sodium alginate solution, caramel solution and physiological saline. In order to prevent the decrease of the circulating blood volume, bleeding and infusion through femoral artery and vein at an average rate of 2 cc/min. were made at the same time and replacement of blood was continued until the rabbit died.

The average blood replacing capacity of Japanese dextran was 93.2 cc/Kg of rabbit body weight, this value was some 1.8 times higher than those of the other three infusion fluids, and the superiority of dextran was clearly shown. The average hematocrit value of rabbits replaced with the dextran was 5.3 at the time of death.

The author concludes that perhaps such excellent results are based on the facts that Japanese dextran gives no bad effect on the physiological function but adversely rather desirable effects on the circulatory system in case of hemorrhage.

Although the author has reported in the first paper of this series that in vitro the efficiency of oxygen saturation of blood decreased slightly when mixed with the dextran, from the results of this experiment it is considered that the same phenomenon does not occur in vivo.

Studies on the Acetone-Butanol Fermentation. Part I. The Vitamin Requirements of Acetone-Butanol Bacteria, Strain 314. (p. *544~548*)

By Shinji DOI, Seinosuke SUGAMA and Munetoshi SHIMIZU

(*Department of Agricultual Chemistry, Faculty of Agriculture, Nagoya University, Anjo*)

To elucidate a chemically defined medium suitable for the production of neutral solvents, the nutritional requirements of *Clostridium* strain 314 have been investigated.

A method to prepare a washed cell-suspension for an active inoculum is described. Strain 314 may be grown luxuriantly in a medium consisting of biotin, *p*-aminobenzoic acid, thiamine hydrochloride, organic bases, salts, glucose and casein acid hydrolyzate as nitrogen sources. Biotin was indispensable for the strain. *p*-Aminobenzoic acid and thiamine hydrochloride were required for the normal fermentation. The optimal pH range was from 5.5 to 6.0.

In this partially defined medium, the normal fermentation took place; glucose was fermented almost completely and the yields of butanol, acetone and ethanol were 20, 7 and 3 per cent, respectively, on the basis of glucose fermented.

Studies on the Acetone-Butanol Fermentation. Part II. The Amino Acid Requirement and Growth Promoting Substance in Casein Acid Hydrolyzate. (p. *548~552*)

By Shinji DOI, Seinosuke SUGAMA, Masayoshi YOKOI and Isamu ADACHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University, Anjo*)

As previously reported, the normal acetone-butanol fermentation was carried out by *Clostridium* strain 314 in the partially defined medium containing casein acid hydrolyzate as nitrogen sources. But this could not be replaced by a mixture of 18 amino acids of approximately the some composition as casein. With amino acids as nitrogen source, growth was retarded and the type of fermentation was changed to the acid type.

The hydrolyzate was fractionated by means of reversible resin columns. Activity was observed exclusively in the acidic fraction containing aspartic acid, threonine, serine, glutamic acid and a small amount of glycine as known amino acids.

With or without this fraction, the amino acid requirements of strain 314 have been investigated from which it was found that isoleucine is indispensable and valine and glutamic acid are most requisite for normal fermentation. Aspartic acid, asparagine, threonine, serine, glycine and alanine showed a slight stimulatory effect. Lysine, arginine, histidine, leucine, phenylalanine, methionine, tryptophane and proline were not required, and cystine and tyrosine were somewhat inhibitory.

Proteolytic Enzymes of Butyl Bacteria. Part I. Changes of Proteolytic Activity in the Acetone-Butanol Fermentation and Purification of the Enzyme. (p. *552~557*)

By Shinji DOI, Yasuyuki KANEKO and Fuji UCHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University*)

Many studies on bacterial protease have been reported, but so far in regard of *Clostridia*, with the exception of the pathogens, few investigations have been made on those of butyl bacteria, useful industrial microorganisms.

This work was undertaken to elucidate tha properties of the proteinases produced by acetone-butanol bacteria, strain 314, and its role on the mechanism of fermentation or bacterial growth.

This organism, when grown in corn mash, excreted proteolytic enzyme into culture medium, activity of which followed the titrable-acidity curve of fermentation mash.

Influences of several factors on the activity were investigated and it was found that its maximal activity lies in the range of pH 5.6–5.8, although maximal activity of other bacteria were usually found to lie in neutral or alkaline pH. The proteinase was purified from culture filtrate of about 20-fold by the application of various fractionation methods.

Proteolytic Enzymes of Butly Bacteria. Part II. The Effect of Calcium Carbonate on the Proteolytic Activity in the Acetone-Butanol Fermentation and Some Properties of the Enzyme. (p. *558~562*)

By Shinji DOI, Yasuyuki KANEKO and Fuji UCHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University*)

Adding excess amounts of $CaCO_3$ to the corn mash medium, proteinase activity of acetone-butanol bacteria, strain 314 increased fivefold. Therefore, this culture was used to obtain enzyme preparation. Partially purified preparation showed single protein bonds on

paperelectropherograms and only these bands liquefied gelatin films.

Optimal pH for casein hydrolysis was about 5.5, the activity very stable at pH 5.0, 4°C, and destroyed profoundly by treatment at 55°C for 10 minutes. Ferrous ion protected this inactivation.

Milk casein was more readily hydrolyzed than bovine hemoglobin by the preparation.

Biochemical Studies on *Collybia velutipes*. Part IV. Relation between the Growth and the Fructification of Fungus. (p. *562～566*)

By Shôji WAKITA

(*Agricultural Institute, Yokohama National University*)

1) Culture and mushroom development:
The mycelia develop a mushroom when they are cultured at 24°, for 17 days and then allowed to be kept in a cool place in the range of 15～17° for 10 days.

2) Relation of culture period (days) and mycelia components:
After one washing, the mycelia were dried in the cold under ventilation to prevent decomposition.
   a. Free-amino acids:
   Free amino acid was tested by a partition chromatography at the early-stage of culture, in the mycelia these existed alanine and arginine exceedingly, aspartic acid, glutamic acid and lysine moderately. As the lapes of culture proceeded these amino acids decreased and α-amino-n-butyric acid, valine and leucine were produsedd.
   b. Non-reduced sugar and free-amino-N:
   When the mycelia were cultured at 24°, at each period, the former decreased exceedingly, while the latter increased after 17 days. Upon being allowed to be kept at 15～17° after 17 days' culture no remarkable change in their contents was observed. When the mycelia developed mushroom both of them decreased remarkably.
   c. Proteins:
   Mycelial extraction by 0.9% sol. was precipitated by cold and pH sifted ethanol. At an early-stage, albumin though it was not ascertained seemed to be present in a considerable amount but decreased rapidly after the development of mushroom.

Studies on the Bulk Sweating of Japanese Native Cigar Tobacco "*Nambu*". Part IV. Changes of the Burning Velocity and the Other Related Qualities of Tobocco Leaves Taking Plase in the Bulk Sweating. (p. *566～569*)

By Isao KANAI

(*Central Research Institute, Japan Monopoly Corporation*)

By using both a mechanical smoking device[1] and a test smoking method, the changes of burning qualities as well as the characters of the residual ash of tobacco leaves occurring in bulk-sweating were studied. "Nambu" leaves were used as the wrapper and "Isabella" leaves were eomployed as the filler and binder. The smoking conditions were: volume of one-puff—50 ml, frequency of puffs—1 puff/30 sec, duration of one puff—2 sec.

During sweating, the increase-rate of the burning velocity of the leaves in the "cutter" position (moisture content—10.0～11.5 %) indicated 11.5～17.0 %, while that of leaves in the "leaf" position was inconsiderable. Furthermore, no significant difference between natural and forced sweating processes was recognized in burning velocity of the latter fermented leaves. It was also found that the color and coherence of ash as well as the area and uniformity of the carbonized zone of the leaves were considerably improved in leaves of the "cutter" position, while they were not improved in those of the "leaf" position.

1) M. Izawa and Y. Kobashi, *J. Agr. Chem. Soc.* Japan, **29**, 754 (1955).

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(In Japanese)
Vol. 32, No. 8 (1958)

Studies on the Aerobic Sugar Metabolism of Yeast. Part I. On the Organic Acid Producing Ability of Various Strains. (p. *571~576*)

By Yuji SASAKI and Shoichi TAKAO

(*Applied Mycological Laboratory, Faculty of Agriculture, Hokkaido University*)

There are a great many reports on the aerobic sugar metabolism of moulds and bacteria.

However, the sugar metabolism of yeasts have been well studied for the anaerobic pathway, namely, alcoholic fermentation, and only a few reports on the oxidative metabolism have been found.

From these points, to make clear the aerobic sugar metabolism of various yeasts, 77 strains of yeasts were cultured in the shallow liquid media containing 10% glucose and these solutions were chiefly examined for organic acids which may be most products from sugar under aerobic conditions.

From the results obtained, it was recognized that without $CaCO_3$ the acidity by every strain was small, and there is little difference among these acidities.

When $CaCO_3$ was added various types of aerobic metabolism were found. For example, several strains produced a large amount of pyruvic acid exclusively, especially *Saccharomyces copsis* accumulated about 22% of this acid based on glucose consumed.

While certain strains produced considerable amounts of other acids except pyruvic acid, particularly several strains of *Candida* sp. isolated from fruits produced a large amount of organic acids but did not accumulated pyruvic acid at all. From these facts, it is considered that the acid will be probably produced by means of the direct oxidation of glucose.

Studies on the Aerobic Sugar Metabolism of Yeast. Part II. Gluconic Acid Production by Yeast. (p. *576~577*)

By Yuji SASAKI and Shoichi TAKAO

(*Applied Mycological Laboratory, Faculty of Agriculture, Hokkaido University*)

In the previous paper, it has been recognized that several strains of *Candida* sp. isolated from fruits produced a large amount of organic acids but did not pyruvic acid. Then, in this experiment the identification and confirmation of the organic acid produced by 6 strains of these *Candida* sp., was carried out.

As a result, it was confirmed that this organic acid was gluconic which has been scarcely reported for yeasts.

And then such a high yield as about 50% based on glucose employed was obtained in still culture for 14 days by the best gluconic producting strain among these yeasts tested. Moreover, it was found that a greater part of the acids produced by these *Candida* sp. was gluconic acid.

The gluconic acid production by certain moulds and bacteria have been well known, but as regards its production by yeast there is a only report published by Walker and Remachandran (1949).

Studies on Pungent Principles of Capsicum. Part I. On Isolation of the Pungent Principles. (p. *578~581*)

By Sadayoshi KOSUGE, Yukio INAGAKI and Kin UEHARA

(*Faculty of Agriculture, Gifu University, Gifu*)

Capsaicin is known as the only pungent principle in *Capsicum annuum* L.. But capsaicin isolated from Japanese capsicum has been found by the authors to be a mixture of two pungent principles. Both principles are isolated from capsaicin by paper partition chromatography, the mixture of 0.1 M–NaOH and 0.05 M–Na_2CO_3 (1 : 1) being used as developing agent, or by liquid chromatography, 0.4 N–HCl saturated with benzene being used as the developing agent and filter paper powder as absorbent. In the paper chromatography, these principles give individually a white opaque absorption band on the half-drying translucent paper. The principles are extracted from the absorption band or the fractions of the developing agent with ether, and are recrystallized from peteroleum ether. One, m.p. 65.9~66.3°C, given R_F 0.68 and other, m.p. 65.6~65.8°C, R_F 0.55 in the above-mentioned paper chromatography by Toyo paper No. 50. Folin-Looney's reagent produces with these principles a rich blue coloration, hence the reaction is employed to detect principles.

Production of Fungal Amylase. Part I. On Fuwa Method of β-Amylase Determination. (p. *581～584*)

By Nobuhiro YAMADA
(*Institute for Fermentation, Osaka*)

The author examined Fuwa method[1] of amylase microdetermination, calculated the order of reaction with current and newly presented methods, and concluded as follows. (1) The hydrolysis of amylose β-amylase was essentially a reaction of the first order as to amylose. However, each curve of the hydrolysis simulated reaction of much higher order. (2) Even though the final volume of the reaction solution was to be diminished to a volume of 10 cc in Fuwa method of β-amylase determination, it was not adequate to modify the concentration of the substrate and the procedure.

1) H. Fuwa, *J. Biochemistry, Japan*, **41**, 583 (1954).

Butanol Fermentation. Part XXII. Fermentation of Molasses. (3) Some Factors Affecting Solvent Production. (p. *585～590*)

By Motoyoshi HONGO and Ken NAGATA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The following factors affecting the acetone-butanol fermentation of molasses were investigated for the industrial use of the isolated strains.

(1) Ammonium sulphate (0.2～0.3 g/100 ml) or ammonium chloride (0.16～0.20 g/100 ml) gave satisfactory results as source of nitrogen.

(2) In general, calcium superphosphate (0.05–0.10 g/100 ml) was effective.

(3) In some strains, addition of soy bean cake (0.1 g/100 ml) was effective for shortenning the fermentation time or increasing the solvent yield.

(4) Addition of distillation slop from alcohol fermentation made the remarkable effect for prevention of foaming and, in some strains, increased the yield. Slopping back was not effective for prevention of foaming and slowed down the fermentation slightly, but tended to increase the yield.

(5) The strains isolated by heat shocking of 80° for 10 min. showed satisfactory results by heat shocking of 100°, 90 sec. too. It was better to repeat this heat shock several times.

(6) Addition of other microorganisms was not effective.

Butanol Fermentation. Part XXIII. Fermentation of Molasses. (4) Control of Temperature. Appendix. Fermentation of Date-palm Fruits. (p. *590～594*)

By Motoyoshi HONGO and Ken NAGATA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The optimum temperature for the acetone-butanol fermentation of molasses by the isolated strains was 30°. At 35°–37° the fermentation time was shortened, but the yield of solvents decreased. So the temperature was controlled as follows: it was maintained at 35°–37° during the propagation of bacteria and at 30° during the production of solvents. By this control, the fermentation time was shortened, especially in high test molasses, without decrease of the yield. And the amount of seed culture in seed developing process was very decreased.

Appendix. Fermentation of Dried Fruits of Date-palm.

This fermentation gave satisfactory results by use of some strains isolated for the fermentation of molasses. The yield of solvents was about 32% in 2 days when the concentration of Date-palm was 6.0–6.5 g/100 ml as inverted sugar. Optimum conditions, e.g. requirements for nitrogen or phosphate, control of temperature etc., were ascertained to be the same as those in the fermentation of molasses.

Studies on the Preservability of Bakers' Yeast. Part I. On the Nitrogen Starvation. (p. *594～598*)

By Masaya HAYASHIBE and Kôtarô MATSUMOTO
(*The Research Department, Oriental Yeast Manufacturing Co., Ltd., Tokyo*)

In order to enhance preservability of bakers' yeast (storage life and stability of fermentation activity during storage), it was attemped to increase carbohydrate content and to decrease nitrogen content. For this purpose, environmental conditions for nitrogen starvation were examined. Time of starvation, nature of starting yeast, presence of nitrogenous compounds in the medium, rate of sugar supply and rate of aeration were greatly influenced on the enhancement of preservability. On the contrary, temperature, pH and presence of bios-components, and of phosphate did not seriously affected on the effect of nitrogen starvation. As regards the variations in chemical composition by nitrogen starvation, the content of TCA (trichloracetic acid) soluble carbohydrate and glycogen always varied remarkably, and the contents of these com-

ponents were significantly correlated with the storage life of yeast. The fermentation activity of nitrogen starved yeast varied according to the contents of amino acids pool of the starting yeast. The increase of fermentation activity after starvation occured in the yeast containing large amounts of amino acids pool and vice verse.

Studies on the Preservability of Bakers' Yeast. Part II. Relation to the Chemical Compositions and Accumulation of Carbohydrates by Oxidative Assimilation. (p. *599~603*)

By Masaya HAYASHIBE

(*The Research Department, Oriental Yeast Manufacturing Co. Ltd., Tokyo*)

Among the variations in chemical compositions caused by nitrogen starvation, the most serious factors influencing upon the preservability of bakers' yeast were discerned. It was also observed in this connection that the accumulation of carbohydrates by oxidative assimilation was greatly influenced by the presence of nitrogenous compounds and phosphate in the medium. Nitrogenous compounds deplete the accumulation of carbohydrates, especially TCA (trichloracetic acid) soluble carbohydrates. Phosphate increased alkali insoluble carbohydrates but had no effect on the TCA soluble carbohydrates. In the course of nitrogen starvation, the preservability and the TCA soluble carbohydrates increased in parallel. As the nitrogen starvation proceeds, the fermentation activity inicially increased and then decreased gradually, and this behaviors of fermentation activity corresponded to the depletion of amino acids pool, that is, most of the amino acids pool was rapidly utilized when the fermentation activity increased. It was concluded that the increase in the contents of reserve carbohydrate intimately related to the enhancement of preservability as a results of detailed investigations on this problem.

Protease Production by the Submerged Culture of *Aspergillus oryzae*. Part II. Selection of Suitable Media for the Production of Protease active in Neutral and Alkaline Reaction. (2) (p. *607~609*)

By Kazuo MOTONAGA and Yukichi MIURA

(*Takamine Laboratory, Sankyo Co., Ltd.*)

In the previous paper[1], the survey of strong protease producer among a number of strains of *Aspergillus oryzae* was conducted and suitable media were investigated. The most suitable medium was different from others in two points:

(i) It contained 3% of potassium phosphate (mono basic).

(ii) Initial pH was 7.5 and the pH was kept neutral and basic during incubation. In the present report, effect of potassium phosphate on the protease production was investigated.

(1) When pH of the medium was maintained almost constant, as shown in Part I[1], sodium sulfate and sodium chloride were able to used in substitution for potassium phosphate showing about same effect to a certain extent.

(2) When pH of the medium was much changeable, for instance protein-containing media, the effect of potassium phosphate seemed to depend upon its pH-buffering action rather than increasing of osmotic pressure caused by high concentration of the salt. In other words, when potassium phosphate was added to the pH-changeable media, pH was kept almost constant and protease production was remarkably increased, while sodium sulfate did not show such effects.

(3) When ion-exchange resin was added to the pH-changeable media, pH was kept almost constant and protease production was considerably increased.

1) K. Motonaga, Y. Miura, *J. Agr. Chem. Soc. Japan*, **32**, 422 (1958).

Studies on the Nucleoprotein in the Embryo of *Zea Mays*. (p. *610~614*)

By Kaneo HAYASHI, Yukio NAGATA, Takashi INOUE and Ichiro YANAGISAWA

(*Biochemical Laboratory, Faculty of Agriculture, Gifu University*)

It was confirmed that the embryos of *Zea Mays* contain pentose nucleoprotein. Albumin and globulin fractions were isolated, and it was found qualitatively that both of them contain a considerable amount of pentose nucleic acid and a slight amount of desoxypentose nucleic acid. The amount of globulin fraction was very little, but that of albumin was considerable. By our paper electrophoretic studies the albumin was proved to be comparatively a homogeneous monocomponent. And from its physical and chemical properties it was presumed that it contains pentose nucleic acid.

Furthermore the nucleic acid was isolated and purified to the protein-free state, and was identified as a ribonucleic acid as follows. (a) Nitrogen and phosphorus contents are 7.0% and 4.2%, respectively. So, the nitrogen/phosphorus ratio becomes 1.7. (b) As orcine-hydrochloric acid test is positive, this substance

is assumed to contain pentose, and ribose is detected with hydrolysate by paper chromatography. (c) This substance has the ultraviolet absorption which exhibits the maximum at 260–265 mμ, minimum at 240 mμ. (d) Adenine and guanine as purine bases are detected with hydrolysate by paper chromatography, but no conclusion has ever been drawn as to pyrimidine bases.

Studies on the Sunlight Flavour of Beer. Part IV. Distribution of Sulfur Compounds and Determination of Volatile Sulfur Compounds (p. *615~617*)

By Yataro OBATA and Hiroyuki HORITSU
(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University*)

Investigation for distribution of sulfur compounds in raw materials for beer and for the balance of each sulfur compound were carried out calorimetrically.

As the result, it is found that total sulfur content in malt is 0.058%, in which water soluble content is 0.046% and total sulfur content in hop is 0.180%, in which water soluble content is 0.154%.

For the content of the total sulfur compound, decrease is found during one day, but after then decrease or increase does not found.

Subsequently, hydrogen sulfide and mercaptan which are both volatile sulfur compounds in beer were determined by Brenner's method.

As the result, hydrogen sulfide does not increase on exposure to sunlight or fluorescence, but volatile mercaptan increases on exposuring slightly.

Moreover, it is made clear that hydrogen sulfide content participates in the taste of beer.

Studies on Flavorous Substancies in Soy Sauce. Part XVI. Flavorous Substance in Raw Soy Sauce. (2) (p. *617~622*)

By Yasuo ASAO and Tamotsu YOKOTSUKA
(*Noda Soy Sauce Co., Ltd.*)

The liquid-liquid chromatographic method was apllied to the study of the acid fractions in raw soy sauce. Several low fattyacids, as reported in the previous reports, were recognized paperchromatographically.

Oxalic, benzoic, vanillic, and syringic acids were identified by melting point determination, paperchromatography, and infrared-spectroscopy. Syringicacid was the first syringyl compounds isolated from soy sauce, and ferulicacid was also identified by paperchromatography.

Discussions on the occurrence of various phenolic compounds in soy sauce are pointed.

Studies on Flavorous Substancies in Soy Sauce. Part XVII. Guaiacyl Components in Soy Sauce. (1) (p. *622~628*)

By Yasuo ASAO and Tamotsu YOKOTSUKA
(*Noda Soy Sauce Co., Ltd.*)

As the raw materials of the guaiacyl ingredients in fermented soy sauce, wheat is more important than soybean. Ferulicacid was identified for the first time as one of the guaiacyl compounds in soy sauce, and its important role in the course of the fermentation of soy sauce was suggested.

Vanillin and ferulicacid were identified already after the cooking of wheat bran, the content of total free phenolic compounds of the material reached to the maximum after twenty four hours of the culture, and both of vanillin and ferulicacid changed into vanillic-acid.

Besides glycosided, lignin was considered to be the precursor of these phenolic compounds.

Chemical Studies on the Autolysis (rigor mortis and rigor off) of Meats. Part II. On the Comparison of Glycolysis of Meats. (p. *628~631*)

By Rinjiro SASAKI and Masao FUJIMAKI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

As it has been observed remarkable differences of glycogen contents in the following meats after slaughter, were examined changes of acid soluble phosphorus compounds during aging of pork, horse and chicken meats.

(1) Horse meat has shown much content of glycogen not only immediately after slaughter but also during aging, compared with the other meats.

(2) The content of acid soluble total phosphorus was almost constant and being lower in order of pork, chicken and horse meats.

(3) From results on changes of inorganic phosphorus, released inorganic phosphorus after hydrolysis for seven minutes and glucose-1-phosphate, it was considered that post mortem changes in chicken meat has proceeded and finished in a short time after slaughter.

(4) In the deamination of adenylic acid, these three meats were found to be different. In horse and chickin meats, deamination has proceeded rapidly from immediately after slaughter and they showed the same type of change as shown in death in agony.

(5) From changes of acid soluble phosphorus compounds, it was considered that the aging process of these meats was different.

Chemical Studies on the Autolysis of Meats. Part III. On the Colorimetric Micro-method for the Determination of Glycoged and on the Existence of Bound Glycoged in Meats. (1) (p. *631~635*)

By Masao FUJIMAKI, Reiko SUZUKI and Nobuhiko ARAKAWA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

(1) A new simple micro-colorimetric method for the determination of glycogen in meats was examined in stead of the usual titration method which needed complicated and long time consuming procedure.

(2) The micro-colorimetric method could be applied to horse meat and beef liver which contained much glycogen, but not being applied to beef and pork, for it was influenced by substances which seemed to interfere the development of color.

(3) It was found to be possible to determine simply and rapidly the contents of glycogen in meats by modified colorimetric method which was combined with a part of titration method (the process of precipitating glycogen in meats by alcohol) and a part of colorimetric method (the process of development of color by reaction of glycogen with sulfuric acid.)

(4) By this modified colorimetric method was determined two types (free and bound) of glycogen in meats, and it was found that bound type of glycogen in meats gradually decreased during aging.

Studies on the Acetone-Butanol Fermentation. Part III. On the Stickland Reaction. (p. *636~640*)

By Shinji DOI and Seinosuke SUGAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University*)

The enzymes concerned in the Stickland reaction of the acetone-butanol bacteria, strain 314 and KN 18, were investigated, comparted with those of *Cl. histolyticum* and *Cl. sporogenes*..

Strain 314, KN 18 and *Cl. histolyticum* H 22 did not perform amino acids interactions. By *Cl. histolyticum*, glycine was metabolized with quantitative evolutions of carbon dioxide and ammonia, and an absorption of molecular hydrogen.

By means of silicic acid column, no volatile acids other than butyrate and acetate were proved on the fermenting corn mashes of strain 314 at the period of maximum titrable-acidity. Furthermore, strain 314 and KN 18 was unable to grow in VF bouillon medium unless glucose was added. Using *Cl. sporogenes*, cells grown in VF bouillon glucose medium showed marked changes in the activities of L-amino acid oxidase and amino acid reductase, compared with those grown in ordinary VS bouillon. Q_{O_2} alanine and Q_{H_2} glycine (or proline) of glucose grown cells decreased to about 50 and 25% respectively of those of non-glucose grown cells, on the other hand pyruvate and L-glutamate dehydrogenases of the former increased.

It is considered that the Stickland reaction does not occur in the acetone-butanol fermentation.

Studies on Submerged Mold Amylase. Part IV. On Air Lift Fermentor. (p. *640~643*)

By Takashi FUKINBARA, Jirô KOBAYASHI, Tôru EGUCHI,

(*Scientific Research Institute, Ltd.*)

Akira ISHIDA, Yasuhiko MIYOSHI and Sadajirô TOBE

(*Noda Shôyu Co., Ltd.*)

Authors designed a new fermentor, and to this fermentor the theory of air lift pump was applied.

This fermentor has a total capacity of 3200 l, and two long air lift pipes are equipped inside of fermentor, and two air lift nozzles are equipped at the bottom of fermentor.

When filtrated air is supplied through the air lift nozzles, the mash in air lift pipe ascends upwards, and then the mash circulates.

As results of submerged culture tests of *Asp. awamori* var. *fumeus* by using this fermentor, the amylase activities of this fermentor were almost equal to those of airation-agitation type fermentor in the same volume of air.

As results of alcohol fermentation tests of these submerged culture liquors using as sacchrifying agent, high alcohol yields were gained.

Strong points of air lift fermentor.

The construction expenses of fermentor becomes cheap, and the electricity for cultivation is saved. It is possible to regulate the culture pH in some degree by changing air volume of air lift nozzles.

Biochemical Studies on the Blast Disease of Rice Plant. Part XI. On the Isolation of a Crystalline Volatile Substance "Oryzarol" from Rice Plant, Possessing a Stimulating Effect on the Germination of Blast Mould Spore. (p. *643~646*)

By Kinjiro TAMARI, Jun KAJI, Nagahiro OGASAWARA and Masamichi SUGA
(*Faculty of Agriculture, Niigata University*)

Previously, Kawamura and Ono* made investigations of the germination of Blast mould spores in a petri dish which was set near by rice plants in a vessel and observed the germination and the appressorium formation of Blast mould spores to be remarkably stimulated by a certain volatile substance of rice plant. From this observation, they considered that this substance, existing in the dew drops on rice plant, should play a significant role when Blast mould spores make an attack on this plant.

The authors pursued this substance and isolated a crystalline volatile substance, possessing a remarkably stimulating effect on the germination of Blast mould spore, from the steam distillate of young rice plants.

This crystalline substance was named "Oryzarol" and $C_{25}H_{44}O_3$ was assined as its molecular formula. Oryzarol melts at 44~46°C and strongly smells like rice straws.

* E. Kawamura and K. Ono, *Agriculture and Horticulture*, **21**, 101 (1956).

Studies on the Components of the Green Leaves of *Ficus carica* L. Part II. On the Antibacterial and Antifungal Activities of Psoralene and Essential Oil. (p. *646~647*)

By Shun-ichi FUKUSHI, Hiroshi TANAKA, (*Department of Agriculture, University of Tottori*) and Hiroyuki HORITSU (*Department of Agriculture, University of Hokkaido*)

In the hope of searching the medicinal components out of *Ficus carica* L., the author examined the antibacterial, antifungal and antiyeasty activities of volatile components of the green leaves of *Ficus carica* L. on six kinds of microbs (*B. subtilis, E. coli, Staph. aureus, Pseud. fluorescens, Asp. oryzae* and *Hans. anomala*.)

The result produced after 72 hours of incubation was as follows:

Psoralene (needle crystals, m.p. 165°) was found to be effective in preventing the gwrowth of *Hans. anomara* and *Asp. oryzae* at a concentration of 1×10^{-5} and of 1×10^{-4}, respectively. But it did not inhibit the growth of bacteria even at a concentration of 5×10^{-3} to 1×10^{-4}.

Essential oil ($[n]_D^{15}$ 1.4854, d_4^{15} 0.9572) did not inhibit the growth of the testing microbs at a concentration of 5×10^{-3}. While phenol fraction of essential oil was found to be effective on bacteria at a concentration of 2×10^{-3} and *Asp. oryzae* and *Hans. anomara* at a concentration of 1×10^{-3}, respectively.

Studies on the Amylolytic System of Black-koji Molds. Part VI. Action of Black-koji Amylase System on Raw Starches, especially Glutinous and Non-glutinous Starches. (p. *648~650*)

By Seinosuke UEDA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka, Japan*)

Glutinous rice starch, corn starch and glutinous corn starch were used as adsorbent for black-koji amylase system. The supernatant liquids were assayed for raw corn starch digestibility, from which the degree of enzymes adsorbed was compared. The method used to determine raw starch digestion was the same as previously reported[1]. The following results were obtained.

(1) The susceptibility of the starch granules to the amylase action is proportional to the adsorption-efficiency of the starch granules for the amylase system.

(2) Raw glutinous starches (millet, corn) are digested easily by saccharogenic amylase fraction alone, while non-glutinous starches (millet, corn) with difficulty.

(3) The susceptibility of malt starch is somewhat greater than that of barley starch.

1) S. Ueda, This Bulletin, **21**, 284 (1957).

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